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GUT MICROBIOTA PERTURBATION INDUCED BY EMERGING DIETARY HABITS: AN *IN VITRO* COLON MODEL TO PREDICT THEIR EFFECTS BY A MICROBIOMIC AND METABOLOMIC APPROACH

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ABSTRACT

Over the past 30 years, unhealthy diets and lifestyles have increased the incidence of noncommunicable diseases and are culprits of diffusion on world's population of syndromes as obesity or other metabolic disorders, reaching pandemic proportions.

In order to comply with such scenario, the food industry has tackled these challenges with different approaches, as the reformulation of foods, fortification of foods, substitution of ingredients and supplements with healthier ingredients, reduced animal protein, reduced fats and improved fibres applications.

Although the technological quality of these emerging food products is known, the impact they have on the gut microbiota of consumers remains unclear.

In the present PhD thesis, the recipient work was conducted to study different foods with the substitution of the industrial and market components to that of novel green oriented and sustainable ingredients. The actions taken were: i) to develop a colonic fermentation protocol using a novel i*n vitro* colon model (MICODE); ii) to compare prototype novel foods (or ingredients) with similar standards or commercial products; and iii) to test the effect of different formulations or processes on prebiotic characteristics.

So far, this thesis included eight representative case studies of the most common substitutions/additions/fortifications in dairy, meat, and vegetable products. The products studied were: (i) a set of breads fortified with polyphenol-rich olive fiber, to replace synthetic antioxidant and preservatives, (ii) a set of Gluten-free breads fortified with algae powder, to fortify the protein content of standard GF products, as alternative to animal protein, (iii) different formulations of salami where nitrates were replaced by ascorbic acid and vegetal extract antioxidants and nitrate-reducers starter cultures, (iv) chocolate fiber plus D-Limonene food supplement, as a novel prebiotic formula, (v) hemp seed bran and its alkalase hydrolysate, to introduce as a supplement in foods as an alternative source of fibers and also bioactives, (vi) milk with and without lactose, to evaluate the different impact on human colonic microbiota of healthy or lactose-intolerants, (vii) lactose-free whey fermented and/or with probiotics added, to be introduced as an alternative beverage, exploring its impact on human colonic microbiota from healthy or lactose-intolerants, and (viii) antibiotics, to assess whether maternal amoxicillin affects the colon microbiota of piglets.

The impact on the human or animal colonic microbiota of the aforementioned products was evaluated as omics data carried out by MICODE colon model and interpreted basing on selected and robust microbiomics and metabolomics indicators, Specifically the effect on the composition and absolute

abundances of bacterial taxa representing the core-microbiota of the human colon with the effect on the volatilome, i.e., molecules resulting from colonic fermentation was considered.

The omics techniques used involved microbiomic (quantitative PCR and sequencing of the 16S-rDNA bacterial gene) and metabolomic (Solid Phase Micro Extraction Gas Chromatography/Mass Spectrometry) analyses.

The *in vitro* MICODE colon model was evaluated by several quality control parameters, including the presence of *Archea* species throughout the fermentation period, the ability of FOS to promote probiotics, as well as investigation of microbiome biodiversity, such as the maintenance of similar indices from baseline to the end points of the fermentation experiments, and the retention of some fecal-derived VOCs at the same time of chromatogram retention.

1. GENERAL INTRODUCTION

During the past 30 years, cardiovascular disease has been more prevalent in most European countries as a result of the development of a diet heavy in animal fats and proteins and low in fiber, as well as bad lifestyle choices (Belc et al., 2019). Dietary balancing based on maximum or minimum levels, where available, of certain food components has been offered by the food industry as a solution in addition to research and current identification of more suitable diets (Mediterranean diet). This tactic has resulted in the reformulation of some items that have altered dietary preferences, the addition of fortified foods, alternative ingredients, or supplements, etc. to the conventional diet, whose prebiotic activities have not yet been fully elucidated. In fact, a prebiotic is now described as "a substrate that is used specifically by host microbes, imparting a health advantage" (Gibson et al., 2017). Prebiotics that are now recognized are carbohydrate-based, but the new definition may also apply to other compounds, including polyphenols and polyunsaturated fatty acids that have been converted to their corresponding conjugated fatty acids. There are many studies on traditionally recognized prebiotics, such as non-digestible oligosaccharides like FOS and GOS, but there is no evidence on emerging prebiotics, such as polyphenols, polyunsaturated fatty acids, amino acids, etc. This is especially true considering dietary reformulations (fortification, supplementation, or replacement with alternative dietary patterns) (Gibson et al., 2017). One of the safest and least expensive ways to increase the nutritional value of a given diet is through food fortification, which involves adding one or more essential nutrients to a product in order to prevent or correct a demonstrated deficiency of one or more nutrients. The main fortifications include folic acid, microelements (iron, iodine, zinc), and vitamins. They are intended to make up for food shortages brought on by socioeconomic conditions in developing countries, unbalanced diets, and special needs in industrialized countries, as well as to address general concerns (vitamins A, D, B). Contrarily, supplementation denotes the incorporation of relatively high dosages of micronutrients in a highly absorbable form and is frequently the quickest method to address a deficiency in certain people or sections of the community. The key supplementation categories are vitamins and co-vitamins, essential minerals, essential fatty acids, essential amino acids, phytonutrients, and enzymes. These are the supplements that people take the most frequently. By disrupting the balance of the microbiota's composition and changing its metabolism, consuming significant amounts of reformulated foods might lead to an unbalance in the gut ecology. It is possible that these alterations are linked to the increased prevalence of cardiovascular disease or metabolic syndrome. Preliminary research has been done on the detrimental

effects of reformulated foods with a lower content of micronutrients and foods with higher energy density on the gut microbiota (Moreira Júnior, et al., 2021). The rise in dietary intolerances to particular food substances, such as gluten and lactose, as well as the common belief that a diet devoid of these substances is better, are both significant contributors to changes in eating patterns. This has caused the spread of goods whose new formulations were made possible by removing the relevant hazardous components. For instance, the formulation of gluten-free products (primarily baked goods and pasta) has included the use of starches, dairy products, gum and hydrocolloids, prebiotics, and their combinations in addition to rice and minor high protein grains such as sorghum, corn, quinoa, and amaranth in order to improve the structure, acceptability, and shelf life of gluten-free baked goods. This is also supported by the increased interest in so-called FODMAPs (fermentable oligo-di-mono-saccharides and polyols). FODMAPs include lactose (naturally found in milk), free fructose (naturally found in pears, apples), fructans (naturally found in rye, wheat, and onions), GOS (naturally found in legumes), and sugar polyols sorbitol and mannitol (naturally found in stone fruits, some vegetables, and fermented foods), which have the property of passing unchanged to the large intestine where they are used by the intestinal microflora to produce gas. Although there is strong evidence for pathologies on the efficacy of eliminating foods rich in FODMAPs, such as in the improvement of subjects suffering from IBS (irritable bowel syndrome), the role of the composition of the intestinal microbiota is not yet clear. Despite the high technological quality of the new products for consumers with food intolerances, it has not yet been clear whether their compositional attributes are related to the biodiversity of the intestinal microflora and its contribution to health. In this sense, the growing interest of the scientific community on the intestinal microbiota and the role it plays in the interaction with the host (both healthy and suffering from specific diseases), has accelerated the evolution of alternative systems to the *in vivo* study on animals. Because there are animal models available, it is possible to study the metabolic functions performed by gut microbes. However, these approaches are hampered by differences between animal and human digestive physiology (Deschamps et al., 2020). To understand how nutrition affects the intestinal microbiota, *in vitro* models are a significant tool. However, it must be acknowledged that the initial models used did not effectively capture the dynamics of the digestive process. Because of this, more sophisticated *in vitro* fermentation models have been created, which are good instruments for allowing the screening of a variety of chemicals, including dietary components, viruses, medicines, hazardous compounds, and obviously novel meals that are possible without being constrained by ethical or accessibility issues, analyze the changes and interactions of the microbiota in gastrointestinal settings.

1.1. Gut Microbiota

Over 100 trillion bacteria live in the human gastrointestinal (GI) tract (Thursby et al., 2017). These microorganisms, collectively referred to as the microbiota, also comprise fungus, viruses, and *Archaea* in addition to bacteria (Fan et al., 2021). The microbiome, which is the collection of all the genes in the gut microbiota, contains far more genes than the human genome. In actuality, the gut microbiome has about 3 million genes, compared to the human genome's 23,000 genes (Valdes et al., 2018). Due to their capacity to extract as well as produce several nutrients and metabolites, such as bile acids, lipids, amino acids, vitamins, and SCFA, the bacteria that live in the human intestine play a crucial role in food digestion. Additionally, the intestinal microbiota serves an immunological function by preventing the growth of pathogenic bacteria, maintaining the integrity and functionality of the intestinal epithelium, and preventing bacterial invasion through the synthesis of antimicrobial peptides and food metabolism (Khosravi et al., 2013). Although the study of the gut microbiota has long piqued the interest of researchers worldwide, the fundamental obstacle has been the challenge of growing these microbes. Thanks to recent advancements in technology, it is now possible to identify and measure the microbes that make up the intestinal microbiota by the study of DNA and RNA that has been directly isolated from feces. The sequencing of rRNA 16S is one of the most used methods for this purpose, which is often based on DNA extraction and amplification of the 16S ribosomal RNA gene (Poretsky et al., 2014; Mizrahi-man et al., 2013).

1.1.1. Gut Microbiota Composition

Over 100 trillion bacteria, or 10-100 times as many as human cells, reside in the human gastrointestinal (GI) tract, as was before mentioned (Thursby et al., 2017). Bacteria are grouped into phyla, classes, orders, families, genera, and species from the perspective of taxonomy. The human intestine has just a few phyla but more than 160 species (Laterza et al., 2016). *Firmicutes* and *Bacteroidetes* make up 90% of the major phyla in the gut microbiota, which also includes *Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia* (Arumugam et al., 2011). More than 200 distinct genera, including *Lactobacillus, Bacillus, Clostridium, Enterococcus*, and *Ruminococcus*, make up the *Firmicutes* phylum, of which *Clostridium* makes up 95%. On the other hand, the phylum *Bacteroidetes* mostly consists of the genera *Bacteroides* and *Prevotella*. The least prevalent phylum, *Actinobacteria*, is mostly represented by the genus *Bifidobacterium* (Arumugam et al., 2011). Since there are numerous elements that might affect the intestinal microbiota and because it differs from person to person, it is impossible to determine an ideal composition for it (Rinninella et al., 2019). Antibiotic use, the host's lifestyle, diet, and cultural practices all have an impact on the composition of the gut microbiota. However, it may be claimed that a rich and extremely diversified microbial population is indicative of a healthy and balanced microbiota.

Additionally, the availability of substrates and host secretions, as well as differences in pH, O2 voltage, velocity of digestion (faster from mouth to cecum while slowing down later), and host secretions all affect the composition of the microbiota in different parts of the gut (Flint et al., 2012). Due to its rapid transit (3-5 hours) and high bile concentration, the small intestine provides a more unfavorable environment for the development of bacteria. The large intestine, on the other hand, possesses environmental conditions that are conducive for microbial colonization because of its slow transits and pH range of neutral to slightly acidic. Because of all these factors, the big intestine is the most populated organ (obligate anaerobic bacteria predominate there) (Flint et al., 2012). Therefore, it is conceivable to state that as one moves down the human gastrointestinal tract, the microbiota exhibits a rising quantitative gradient and a diminishing qualitative gradient, with the aerobic bacteria eventually disappearing and being replaced by purely anaerobic bacteria. Numerous studies conducted over the past few years have shown a connection between intestinal microbiota dysbiosis and non-communicable diseases like gastrointestinal conditions like irritable bowel syndrome (IBS) (Bhattarai et al., 2017), cardiovascular diseases (Tang et al., 2019), obesity (Pascale et al., 2019), diabetes (Pascale et al., 2019), cancer (Raza et al., 2019), and neurological disorders (Cryan et al., 2020). Additionally, the so-called "gut-brain axis," or the interactions between the brain and gut microbiota, has recently piqued the interest of scientists, and some investigations have revealed that these interactions are enabled by immune activations and bidirectional neuroendrocrine communication (Mayer et al., 2015), as well as neurological conditions such autism spectrum disorders (Oh et al., 2020) and Parkinson's disease (Hill-Burns et al., 2017). Although the cause or effect of a particular disease may modify the gut microbiota's composition, the correlation between a diversified and rich microbiota and host health status has been shown to be beneficial (Rinninella et al., 2019). The food undoubtedly has a significant impact on the modulation of the intestinal microbiota among other factors, both by directly affecting the host's homeostasis and biological processes and by causing the microbial fermentation of nutrients, such as SCFA, to produce metabolites (Gentile et al., 2018). The functioning of the intestinal barrier and the immune system can be impacted by changes in dietary habits, which can disrupt the mutualistic interaction between the intestinal microbiota and the host.

1.1.2. Gut Microbiota Enterotypes

Although nowadays under debate, a classical concept of human gut microbiome studies is that everyone has different "enterotypes" of intestinal microbiota that can be categorized (Arumugam et al., 2011). Three enterotypes are defined based on the dominant bacterial group: *Bacteroides* (enterotype I), *Prevotella* (enterotype II), and *Ruminococcus* (enterotype III). Different bacterial

genera define each enterotype (**Table 1**). The three enterotypes differ from one another in terms of their unique roles as well as the bacteria that make them up. Each enterotype defines a method for producing energy starting with the fermentable substrates present in the colon through its groups of bacteria and functional properties. In actuality, the enterotype I bacteria mostly obtain their energy from the fermentation of carbohydrates via the glycolysis and pentose phosphate pathways. On the other hand, the glycoproteins of the intestine's mucosal layer can be degraded by the bacteria of enterotypes II and III. Finally, it appears that eating habits are the key criteria used to define enterotypes.

Table 1. Microbiota enterotypes (Arumugam et al., 2011)

Enterotype	Gut microbiota composition	
I	*Clostridiales* *Alkaliphilus* *Lactobacillus* *Catenibacterium* *Bacteroides*	*Parabacteroides* *Slackia* *Geobacter* *Methanobrevibacter smithii*
II	*Veillonella* *Ruminococcaceae* *Holdemania* *Peptostreptococcaceae* *Staphylococcus* *Leuconostoc*	*Prevotella* *Eggerthella* *Desulfovibrionaceae* *Escherichia* *Shigella* *Akkermansia muciniphila*
III	*Ruminococcus* *Staphylococcus* *Marvinbryantia* *Symbiobacterium* *Ruminococcaceae*	*Dialister* *Sphingobacterium* *Gordonibacter* *Akkermansia muciniphila*

1.1.3. Incidence of Dietary Habits

Although the microbiome of a healthy person is generally stable, the microbiota changes in response to lifestyle and diet (Leeming et al., 2019). *Prevotella* and *Xylanibacter* predominated in the intestinal microbiota of African children, while *Shigella* and *Escherichia* were underrepresented, according to a well-known study (De Filippo et al., 2010) that compared the intestinal microbiota of European children, who consumed a Western diet, and children from Burkina Faso, whose diet was high in

local cereals and vegetables and low in fat and animal proteins. On the other hand, a study by Schnorr et al. (2014) compared the gut microbiota of Italian and Hadza subjects. The results showed that *Proteobacteria* and *Spirochaetes* were more prevalent in the African microbiota than *Actinobacteria*, which are a significant part of the Italian microbiota but almost nonexistent in the Hadza microbiota. On the other hand, *Actinobacteria* are extremely rare in the African microbiota. At the genus level, Hadza's intestinal microbiota was found to be particularly rich in *Prevotella, Eubacterium, Oscillibacter, Butyricicoccus, Sporobacter, Succinivibrio* and *Treponema* and on the contrary, it was poor in *Bifidobacterium, Bacteroides, Blautia, Dorea, Lachnospiracterium, Faecalibacterium, Roseburia, Ruminococcus* and *Erysipelotrichaceae* unclassified. Both studies demonstrated that the *Prevotella* enterotype (enterotype II) defined the African gut flora. In fact, the African diet is rich in vegetables and cereals while being deficient in items with animal origin, which promotes the degradation of mucins in concert with *Desulfovibrionaceae*. The European gut microbiota, on the other hand, possessed a *Bacteroides* enterotype (enterotype I). The Western diet, or European diet, is high in animal fats and proteins, which encourages the fermentation of carbohydrates and proteins to produce energy (Ramos et al., 2021). What has been said thus far was also supported by the study of David et al. (2014). According to this research, a diet high in animal products and low in fiber encouraged bile-tolerant bacteria (*Alistipes, Bilophila,* and *Bacteroides)* and decreased the levels of *Firmicutes*, which digest dietary fiber (*Roseburia, Eubacterium rectale,* and *Ruminococcus bromii*). Furthermore, it has been seen that microbial metabolism switches from fermenting carbohydrates to proteins or vice versa when changing the type of diet.

1.1.4. Gut Microbiota Variations in Health and Disease

Age, ethnicity, lifestyle, and diet all affect the composition of the intestinal microbiota, and these physiological changes can have an impact on both intestinal and non-intestinal illnesses. Even if it is still unclear whether dysbiosis is the cause or result of illnesses, it is defined precisely as a change in the gut microbiota's composition. In fact, it is frequently difficult to determine if a change is good or bad, but in any event, persistent external stimulation can harm an ecosystem that is not organized like the microbiota. Below, we'll discuss how changes in the composition of the gut microbiota relate to different diseases.

1.1.4.1. Irritable Bowel Syndrome (IBS)

Irritable bowel syndrome (IBS) is undoubtedly one of the gastrointestinal (GI) conditions that is most prevalent (Bhattarai et al., 2017). In general, there were trends at the phylum level for patients with IBS to harbor a higher abundance of *Firmicutes* and a lower abundance of *Bacteroidetes* compared

to those for healthy controls and thus an increased *Firmicutes:Bacteroidetes* ratio was identified (Labus et al., 2017; Zeber-Lubecka et al., 2016; Nagel et al., 2016; Chung et al., 2016). As the predominant class within the phylum *Firmicutes, Clostridia* were present at higher levels in IBS patients (Labus et al., 2017; Zeber-Lubecka et al., 2016; Nagel et al., 2016) and, at the order level, a coincidentally higher abundance of *Clostridiales* was observed (Tap et al., 2017; Zeber-Lubecka et al., 2016; Nagel et al., 2016). Within the order *Clostridiales*, we noted that conflicting results were reported on the presence of the family *Ruminococcaceae* with a high frequency in the included studies, with increases (Zeber-Lubecka et al., 2016; Carrill et al., 2012) and decreases (Pozuelo et al., 2015; Carroll et al., 2012; Durban et al. 2012) observed in patients with IBS. Although only one study (Labus et al., 2017) found that patients with IBS had higher amounts of the class *Bacilli*, a member of the *Firmicutes* family like the class *Clostridia*, its lower taxon *Lactobacillales* was regularly found to be elevated in patients (Labus et al., 2017; Ringel-Kulka et al., 2016; Carroll et al., 2012).
One of the most well-known probiotics, *Lactobacillus*, has been found in higher abundance in studies (Ringel-Kulka et al., 2016; Carroll et al., 2012). It is also found in higher abundance at the family and genus levels. Patients with IBS have reduced proportions of the *Bacteroidetes*, the second-most prevalent phylum in the human gut. Patients also had lower levels of *Bacteroidetes* at the class level and *Bacteroidales* at the order level, which is consistent with this conclusion (Labus et al., 2017; Nagel et al., 2016; Jeffery et al., 2012). *Prevotella* levels in IBS patients were shown to be both higher (Gobert et al., 2016; Liu et al., 2016; Chung et al., 2016) and lower (Tap et al., 2017). *Proteobacteria*, another significant phylum found in the human gut, have been reported to be more prevalent in IBS-related research (Gobert et al., 2016; Nagel et al., 2016; Chung et al., 2016; Carrolle et al. 2012). Patients with IBS had greater amounts of the phylum's family *Enterobacteriaceae* and order *Enterobacteriales* in particular (Jeffery et al., 2012; Carroll et al., 2012;). *Actinobacteria*, on the other hand, showed both elevated (Nagel et al., 2016; Ringel-Kulka et al., 2016; Jeffery et al., 2012) and decreased (Chung et al., 2016) levels in individuals with IBS, unlike the three phyla stated above.

1.1.4.2. Inflammatory Bowel Disease (IBD)

All disorders characterized by idiopathic, persistent, and recurrent inflammation of the gastrointestinal tract are collectively referred to as "inflammatory bowel disease (IBD)," including Chron's disease and ulcerative colitis (UC) (CRD). According to a study by Frank et al. (2007), *Lachnospiraceae* and *Bacteroidetes* were less common while *Proteobacteria* were more prevalent in IBD patients than in healthy ones. Particularly, there are differences between the effects of UC and CRD on the gut flora. According to Machiels et al. (2014), while butyrate-producing bacteria like

Roseburia hominis and *Faecalibacterium prausnitzii* are less prevalent in CU patients than in healthy controls, the opposite is true for patients with CRD, who have more of the bacterium and less diversity in their microbiota overall (Hansen et al., 2012). Another study (Sokol et al., 2008) found that *Ruminococcus gnavus* increased while *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis* decreased in CRD patients. In this study, it was also demonstrated that giving oral probiotics containing *F. prausnitzii* to patients with CRD can rebalance their microbiota, restoring it to a state of eubiosis, and lessen the severity of their colitis. The loss of balance in the composition of the intestinal microbiota plays a critical role in the severity of the disease, but these studies do not demonstrate a cause-and-effect relationship between the dysbiotic condition of the intestinal microbiota and IBD.

1.1.4.3. Celiac Disease (CeD)

Celiac disease is a chronic autoimmune disease that causes an immune reaction of the body following the intake of gluten. If left untreated, this immune response causes inflammation in the small intestine, which is why it is referred to as a chronic inflammatory bowel disease. Environmental elements, like as the gut flora, also significantly contribute to the development of celiac disease in addition to genetic predisposition (Chander et al., 2018). The etiology of celiac disease is directly related to alterations in the intestinal microbiota; in particular, a connection between the inflammatory state and dysbiosis has been emphasized in celiac individuals (Marasco et al., 2016). The studies of fecal samples and duodenal biopsies in CeD patients on GFD versus GD and normal healthy population showed an alteration of gut microbiota. Comparing CeD patients on GD to the normal population, it was found that the presence of *Bacteroides-Prevotella, Clostridium leptum, Histolitycum, Eubacterium*, and *Atopobium* was higher while the presence of *Bifidobacterium spp., B.longum, Lactobacillus spp., Leuconostoc, E. Coli*, and *Staphylococcus* was lower (Di Cagno et al., 2011; Nistal et al., 2012; Bodkhe et al., 2019; Nistal et al., 2012; Golfetto et al., 2014). When CeD patients received GFD treatment, the elevated microbial concentration decreased to that seen in the general population, indicating that diet affected gut microbiota. However, the majority of research found that when CeD patients were placed on a GFD, the microbiota was only partially restored (Bascunan et al., 2019; Caminero et al., 2019; Bonder et al., 2016). Additionally, several of these patients had CeD symptoms even while on GFD, and their lab results revealed a relative abundance of *Proteobacteria* and a decrease in *Firmicutes* and *Bacteroides*, pointing to dysbiosis as the possible source of their ongoing GI symptoms even on GFD (Wacklin et al., 2014). Although the exact cause of GFD's failure to restore the microbiota to that of healthy patients is unknown, it is possible that individual genetics or the prebiotic effect of GFD may be to blame (Wacklin et al., 2014; De Meij et al., 2013). Although

no cause or effect relationship can be deduced from these studies, the consensus is that dysbiosis may contribute to CeD.

1.1.4.4. Colorectal Cancer (CRC)

Colorectal cancer (CRC) is the fourth cause of cancer death worldwide, with nearly 1.8 million new cases and 881,000 deaths in 2018 (Bray et al., 2018). A study by Wang et al. (2012) found an imbalance in the gut microbiota of subjects with CRC compared to the microbiota of healthy subjects, with a greater abundance of *Bacteroides fragilis, Enterococcus, Escherichia / Shigella, Klebsiella, Streptococcus* and *Peptostreptococcus* and a lower abundance of butyrate producers, such as *Roseburia* and *Lachnospiraceae*, while the healthy control appeared enriched in *Bacteroides vulgatus* and *Bacteroides uniformis*. *Fusobacterium spp.* and colorectal cancer were linked in a study by Kostic et al. (2012) using genomic analysis. The participants with CRC, in particular, displayed higher levels of *Fusobacterium spp.*, in contrast to the pyhla *Bacteroidetes* and *Firmicutes*, which were lacking. Additionally, it was discovered that *Fusobacterium spp*. may influence tumor formation via an inflammatory-mediated mechanism (Kostic et al., 2012), however more research is required in this area.

1.1.4.5. Obesity

Studies on animals have revealed a special connection between obesity and the composition of the gut microbiota, but no such link has been discovered in studies on humans. However, the composition of the human and mouse microbiotas is comparable, with *Firmicutes* and *Bacteroidetes* equally predominating in both (Ley et al., 2005). Ley et al. (2005) discovered that despite all mice being fed a diet high in polysaccharides, obese mice had a relative abundance of *Bacteroidetes* that was 50% lower and a larger abundance of *Firmicutes*. While *Bacteroidetes* have been associated with an adequate body weight, *Firmicutes* with obesity. *Bacteroidetes* have a positive correlation with reduced body fat (Ley et al., 2005), whereas *Firmicutes* and obesity may be associated with increased energy harvesting. In fact, *Firmicutes* possess a greater number of carbohydrate metabolism enzymes, which contribute to the metabolisation of this macronutrient allowing for greater energy absorption (Ibrahim et al., 2012). The genus *Lactobacillus* belongs to the phylum *Firmicutes* and an increase in this genus has been associated with obesity (Ibrahim et al., 2012). Among the bacteria of this genus, *Lactobacillus reuteri* (Million et al., 2013; Munukka et al., 2012) has been correlated with higher BMI. However, despite the association between *Lactobacillus* and obesity, it appears that some bacteria (*Lactobacillus paracasei* and *Lactobacillus plantarum*) of this phylum have a protective effect against weight gain. *Fusobacteria* and *Fusobacterium* are opportunistic pathogenic bacteria

and increase in individuals with obesity. This result was also found by Gao et al. (2017). Overall, the research described above show a connection between changes in the gut microbiota and obesity, along with a decrease in the diversity and differences in the number of genes involved in metabolism. In fact, the microbiome is also impacted by changes in the intestinal microbiota's composition, which allows it to absorb more energy from the diet (Turnbaugh et al., 2006).

1.1.4.6. Type 2 Diabetes (T2D)

Compared with the normal people, the number of *Bifidobacteria*, *Clostridium* and *Firmicutes* in the intestinal flora of diabetics decreased significantly (Larsen et al., 2010; Zhang et al., 2013; Karlsson et al., 2013; Sato et al., 2019), the number of *Bacteroides* (Qiu et al., 2019) and *β-proteus* increased significantly, and the ratios of *Bacteroides/Firmicutes* and *Firmicutes/Clostridium* were positively correlated with blood glucose level, however the ratios appeared to be independent of the weight, confirming that they were associated with reduced glucose tolerance. Wu et al. (2010) found that the bacillus content in the feces of diabetics was well below that of healthy people. LKA et al. (2012) found that the number of *Bifidobacteria* in intestine of patients with T2DM was significantly lower than that of healthy people, while the number of *Enterococcus* fecal was higher than that of healthy people. In early and severe stages of T2DM patients, *Verrucomicrobium* showed a considerable drop, according to Zhang et al. (2013), suggesting that it may be a potential indicator of the disease. When Qin et al. (2012) studied the feces of diabetics, they discovered that these individuals had a moderate intestinal ecological disorder. As a result, butyric acid-producing *Rothia* and *Fecalibacterium prausnitzii* dropped, but potentially harmful bacteria including *Bacteroides stercoris* and *Clostridium ramosum* grew. According to Eckburg et al. (2005), diabetics had sharply higher levels of conditional pathogenic bacteria like *Bacteroides, Escherichia coli*, and *Desulfovibrio* while healthy subjects had higher concentrations of butyric acid-producing bacteria like *Clostridium, Eubacterium rectale*, and *Fecalibacterium prausnitzii*. In these patients, the intestinal flora's cell membrane was more active in the transport of sugars and branched-chain amino acids, but the production of butyric acid decreased, and the proinflammatory response to oxidative stress increased.

1.1.4.7. Lactose intolerance

Lactose intolerance refers to gastrointestinal symptoms related to incomplete digestion of lactose. The study by He et al. (2005) found by FISH that the composition of the fecal microbiota was not different between lactose-tolerant and lactose-intolerant subjects, although differences in metabolic activities were found. This could be because the detection of bacteria by FISH is not based on a strain-specific level but by genus or group. Bacterial strains of the same genus or group may have different

metabolic capacities. In addition, the limit of detection of bacteria in feces with FISH is 106-107 cells/g feces (0.001-0.01% of total fecal bacteria). Bacterial groups whose numbers are below this level cannot be detected with FISH. In addition, wide variations in the number of bacteria between individuals are often reported, making it difficult to elucidate differences in bacterial composition. Similar results to those of He et al. (2005) were also observed by the study of Zhang et al. (2004), which determined the relative amounts of various bacterial groups in fecal samples from healthy and lactose-intolerant subjects, calculated as a percentage of the DAPI count. The predominant groups were *Eubacterium rectale/Clostridium coccoides* and *Bacteroides/Prevotella* but with no significant differences between the two groups.

1.1.5. Interplays Between Food Components and Gut Microbiota

1.1.5.1. Carbohydrates and Gut Microbiota

Digestible and indigestible carbohydrates are differentiated. The latter undergo enzymatic digestion in the small intestine and then enter the circulation as glucose. Examples of the former include glucose, fructose, and galactose. Contrarily, the other, also referred to as "dietary fiber," withstands the activity of digestive enzymes in the small intestine and makes it to the colon intact. Lignin, resistant starches, and non-digestible oligosaccharides including raffinose, stachyose, oligofructose, and inulin are a few examples of non-digestible carbohydrates (Mudgil et al., 2013). Dietary fibers are further classified as fermentable or not, as well as soluble or not in water. While non-fermentable fibers like cellulose, hemicellulose, lignin, and resistant starch are insoluble in water, fermentable fibers like inulin, pectin, beta-glucan, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) are soluble in water (Galanakis, 2019). The intestinal microbiota can utilize fermentable fibers as compared to non-fermentable fibers when it comes to the interaction between carbohydrates and the microbiota (Galanakis, 2019).

1.1.5.2. SCFAs

The intestinal microbiota ferments the undigested dietary fibers in the colon, which results in the generation of monosaccharides, SCFA (Acetic acid, Butyric acid, and Propionic acid), and gas (CH3 and CO2) (Blaak et al., 2020). Acetic acid then acts as a substrate for the synthesis of cholesterol, Propionic acid participates in glucose metabolism (Levy et al., 2016) while Butyric acid plays a key role in maintaining the barrier function (Kelly et al., 2015). The roles of the SCFA, however, go well beyond what has been mentioned. They also support colon homeostasis by promoting epithelial cell differentiation and proliferation, appropriate water absorption, and the preservation of the mucosa's

integrity (Martin-Gallausiax et al., 2021). Additionally, SCFAs have been demonstrated to function as anticancer agents while simultaneously contributing to epigenetic control (Gentile et al., 2018). The composition of the gut microbiota and the amount of carbohydrates added to the diet have a major impact on the amount and quantities of the different SCFAs that are generated (Nylund et al., 2016). In light of this, the kind and quantity of fermentable dietary fiber in a person's diet also affects the bacterial composition of their stool (Maukonen et al., 2015).

1.1.5.3. Prebiotics

Prebiotics are defined as being selectively used by host microorganisms conferring a health benefit (Gibson et al., 2017). Although the currently recognized prebiotics are carbohydrate-based, the new definition may also apply to other substances including polyphenols and polyunsaturated fatty acids that have been converted to their corresponding conjugated fatty acids. In particular, the carbohydrates that the intestinal microbiota can utilize are those that withstand the host's enzymatic digestion and make it to the colon intact (Sonnenburg et al., 2016). According to Sonnenburg et al. (2016), an increase in *Bacteroides thetaiotaomicron*, which breaks down intestinal mucus, is caused by a diet low in carbohydrates. When fermentable carbohydrates are scarce, these bacteria can utilise the glycans present in the host's mucus, weakening the intestinal barrier (Mu et al., 2017). According to a study by Singh et al. (2018) using a mouse model, inulin can change the intestinal microbiota's composition by decreasing *Firmicutes (Roseburia, Clostridium cluster I, IV,* and *XIV*) and increasing *Bifidobacterium spp*. and *Bacteroidetes*. Vandeputte et al. (2017) demonstrated the influence of inulin on the intestinal microbiota, in particular on the relative abundance of *Anaerostipes*, *Bilophila* and *Bifidobacterium*.

1.1.5.4. Proteins and Gut Microbiota

Fermentation of amino acids takes place in the distal colon by major microbial phyla including *Firmicutes, Bacteroidetes* and *Proteobacteria*. Proteolytic fermentation also produces SCFA, even if in smaller quantities than carbohydrate fermentation, along with BCFA (such as Isobutyrate, 2-Methyl butyrate and Isovalerate) and potentially toxic substrates such as Nitrosamines and Trimethylamine N-oxide (Scott et al., 2013). According to a study by Scott et al. (2013), *Escherichia*, *Pseudomonas*, *Proteus*, and *Klebsiella* all produce Nistrosamine. Depending on the protein type, proteins have different impacts on the gut flora. For example, animal proteins, especially those from red meat and dairy products, might increase the number of bile-tolerant bacteria like *Bacteroides*, *Alistipes*, and *Bilophila* (David et al., 2014). Trimethylamine N-oxide (TMAO), a substance linked to the development of cardiovascular disorders and renowned for its proaterogenic potential, is

produced more frequently as a result of these changes. Inflammatory bowel diseases (IBD) were more likely as a result of the bacteria that reduce sulfates (such as *Desulfovibrio spp*.) producing hydrogen sulfide (H2S) from the sulfur amino acids (such as Methionine, Cysteine, and Taurine) that constitute proteins of animal origin (Jantchou et al., 2010). Additionally, a study by Swiatecka et al. (2011) revealed that the fermentation of animal-derived proteins decreases *Bifidobacterium* abundance and SCFA production, raising the risk of developing IBD (Singh et al., 2017). However, the same author has also demonstrated that eating proteins with a vegetable origin, such those in peas, boosts the health advantages of *Bifidobacterium* and *Lactobacillus* while lowering levels of the diseases *Bacteroides fragilis* and *Clostridium perfringens* (Swiatecka et al., 2011). Additionally, a hamster model showed that the addition of soy protein concentrates to a Western diet boosted levels of *Bifidobacteriaceae* and *Clostridiales* while decreasing levels of *Bacteroidetes* (Butteiger et al., 2016). The beneficial effect of soy consumption on the intestinal microbiota could be further enhanced by soy isoflavones (Vazquez et al., 2017), while this benefit could be counterbalanced by a detrimental effect of soy saponins on the intestinal barrier (Miao et al., 2018). Finally, the fermentation of plant proteins may be associated with an increase in the abundance of *Bifidobacterium* and *Lactobacillus*, stimulating the production of SCFA (Singh et al., 2017).

1.1.5.6. Fats and Gut Microbiota

Fats consumed as part of the diet have an impact on the composition of the gut microbiota (Candido et al., 2018). In general, the classification of dietary fatty acids into saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids is based on the presence of double bonds between the carbon molecules. No changes were found in the *Firmicutes:Bacteroidetes* ratio in the cecum of either the mice fed HFD or LFD. However, HFD increased the alpha diversity in both cecum and colon compared to LFD, and the *Firmicutes:Bacteroidetes* ratio was significantly decreased in mice fed HFD (Wang et al., 2020). Therefore, a high fat intake, especially of SFA, can result in an intestinal dysbiosis condition that may change the intestinal barrier. Indeed, patients who regularly consume a lot of dietary fat have a predominance of sulfate-reducing bacteria (SRB) in their gut microbiota (Jissennagger et al., 2016; Johansson et al., 2008). The abundance of these bacteria, including *Bilophila wadsworthia*, results in a high concentration of sulfide being produced, which can reduce disulfide bonds in the mucus, thus causing lysis of the secreted MUC2 (oligomeric gelling mucus) polymer network and weakening the mucus layer's stability (Devkota et al., 2013). According to several research (Devkota et al., 2013, Gruber et al., 2013, and Devkota et al., 2015), a diet high in saturated fat can encourage the growth of SRB, decrease the mucus layer, worsen the condition of intestinal inflammation, and lead to the development of colitis and IBD.

MUFAs, including Oleic acid which is rich in extra virgin olive oil (EVOO), are abundant in the Mediterranean diet. Colica et al. (2017) have shown that many of the cardioprotective properties of the Mediterranean diet are linked to the consumption of extra virgin olive oil and for this reason its consumption is recommended to hinder the onset of coronary heart disease (Colica et al., 2017). However, it appears that its phenolic components, not its Oleic acid concentration, are what give it its anti-inflammatory and antioxidant benefits (Bulotta et al., 2014). It appears that a diet high in MUFA has no effect on the richness/diversity indices, the distribution of the phylum, or the *Bacteroidetes/Firmicutes* ratio in terms of the composition of the gut microbiota. However, diets high in MUFA appear to increase the genera *Parabacteroides, Prevotella,* and *Turicibacter* and the family of *Enterobacteriaceae* while decreasing the genus *Bifidobacterium* at the family and genus levels (Wolters et al., 2018).

The foods highest in PUFA are oils from sunflower, soybean, corn, nuts, and seeds. Omega-3 PUFAs, which include Linolenic acid, and omega-6 PUFAs are the two main types of PUFAs (including Linoleic acid). Since the human body is unable to generate PUFAs, they are also referred to as "essential fatty acids" and must be consumed through diet. The gut microbiota can be encouraged to have a healthy composition by consuming foods high in omega-3 PUFAs, which also boost the production of anti-inflammatory substances. According to several research (Watson et al., 2018; Noriega et al., 2016; Menni et al., 2017), omega-3 PUFAs can improve butyrate production by promoting the growth of *Lachnospiraceae* taxa and restoring the *Firmicutes / Bacteroidetes* ratio to appropriate levels (Watson et al., 2018; Noriega et al., 2016; Menni et al., 2017). Due to the influence of the intestinal microbiota, a high omega-6 / omega-3 PUFA ratio, a feature of the Western diet, is linked to increased intestinal barrier permeability and plasma concentrations of LPS (Kaliannan et al., 2015). Conjugated linoleic acids (CLA) are a different class of PUFA, with 18: 2cis-9, trans-11 (9,11 CLA or Romanian acid) and 18: 2trans-10, cis-12 standing out (10,12 CLA). This PUFA family originates from the partial biodegradation of Linoleic acid that is catalyzed by isomerases, bacteria-produced enzymes that are abundantly present in milk, dairy products, and the fat of ruminant animals. The Food and Drug Administration (FDA) classifies CLAs as "generally safe (GRAS)" and attributes them with antiatherosclerotic, antiobesogenic, and anticancer activities (Den Hartigh et al., 2019). Animal studies have demonstrated that adding 10,12 CLA to the diet encourages species-level increases in *Butyrivibrio, Roseburia,* and *Lactobacillus* as well as phylum-level decreases in *Firmicutes* and increases in *Bacteroidetes*, with an associated rise in fecal butyrate concentration and plasma acetate concentration (Marques et al., 2015; Den Hartigh et al., 2018). The positive benefits of CLA might be at least partially explained by these positive effects on the composition of the gut bacteria.

1.1.5.7. Food Additives and Gut Microbiota

Due to the spread of ultra-processed foods especially in the Western diet, the number of food additives, such as sweeteners and emulsifiers, which are used by the food industries, has increased considerably (Carocho et al., 2014). For example, artificial sweeteners are utilized to improve food taste and texture as well as stability. Several studies (Spencer et al., 2016; Suez et al., 2014) have shown how sweets can alter the composition of the intestinal microbiota and encourage the beginning of effects, demonstrating the detrimental influence of sweeteners on the microbiota. In fact, Suez et al. (2014) have established a relationship between the disruption of microbial metabolic pathways caused by artificial non-caloric sweeteners (NAS) and the host's propensity to acquire metabolic disease. In this study, saccharin was administered for one week to a group of participants who did not typically consume NAS. After this injection, their glucose tolerance decreased. The NAS responders' feces were examined, and the results showed a rise in *Bacteroides spp*. and *Lactobacillus spp*. and a fall in *Clostridiales spp.* Several studies (Spencer et al., 2016; Roca-Saavedra et al., 2018) have shown that the intake of NAS increases *Bacteroides* and some *Clostridiales spp*., and decreases some *Clostridiales spp., Bifidobacterium* and *Lactobacillus*. A study in mice given low doses of aspartame in water (5-7 mg / day) showed that this administration resulted in an increase in *Enterobacteriaceae* and *Clostridium leptum* along with elevated fasting glucose levels and altered insulin responses (Palmnas et al., 2014). These results raise the issue of the widespread use of artificial sweeteners and the function of the gut microbiota because these changes in the microbiota's composition may result in glucose intolerance. Compared to what has been observed for other low-calorie sweeteners, stevia-based sweeteners (i.e., extracted from the stevia leaf) did not show the same effects on anaerobic fecal cultures from healthy individuals (Magnuson et al., 2016). Food emulsifiers, such as lecithins and mono- and diglycerides of fatty acids, could also harm the host in addition to natural sweeteners, for example, by encouraging bacterial migration through epithelia *in vitro* and causing inflammation, or systemic, by changing the localization and composition of the microbiota (Chassaing et al., 2015). A study by Chassaing et al. (2015) showed that the intake of emulsifiers reduces intestinal microbiota diversity, decreasing *Bacteroides* and increasing *Akkermansia muciniphila, Proteobacteria* and mucolytic species, including *Ruminococcus gnavus*. These microbiota alterations have led to dysbiosis and chronic intestinal inflammation, promoting the onset of colitis and metabolic syndrome (Chassaing et al., 2015).

1.1.5.8. Nitrates and nitrites

Red and processed meats have been classified as possibly carcinogenic (Group 2A) and carcinogenic (Group I), respectively, by the International Agency for Research on Cancer (IARC), a research arm of the World Health Organization (WHO) (Jiang and Xiong, 2016). The inclusion of nitrates and nitrites is a known drawback of processed meats. These additives serve a variety of purposes, such as preventing lipid oxidation, preserving color, and maintaining microbiological safety by inhibiting pathogens (Majou and Christieans, 2018), but in recent years they have come under fire for their capacity to produce cancer-causing N-nitrous compounds. In fact, the human colon contains amines and amides from the bacterial metabolism of amino acids, and these substances may be N-nitrous in the presence of nitrosylated heme from unabsorbed red meat residues (Herrmann et al., 2015; Johnson, 2017; Meurillon and Engel, 2016). Some research has attempted to season meat without nitrites, but the product was of poor organoleptic and microbiological quality, yielding disappointing results (Hammes, 2012). Because plant extracts include a significant amount of polyphenols, which are recognized for their antioxidant and antibacterial characteristics, many research have sought to investigate the usage of plant extracts as alternatives for nitrates and nitrites (Jiang and Xiong, 2016; Shah et al., 2014; Shan et al., 2009; Pini et al., 2020). However, none of these studies focused on the effect of these alternative formulations on the gut microbiota.

1.1.5.9. Polyphenols and Gut Microbiota

Foods such as fruits, vegetables, medicinal plants, microalgae, herbs, seeds and grains, and beverages such as coffee, tea, cocoa, and wine are rich in polyphenols, of which more than 10,000 have been identified (Li et al., 2014). In recent years, the scientific world has focused on these chemicals since they may help prevent disorders including cardiovascular disease, diabetes, obesity, and many others (Scalbert et al., 2005), even though it is unclear how well they are absorbed and bioavailable in people. However, it is evident that a critical factor in the bioavailability of these chemicals is the gut flora (Ozdal et al., 2016). In general, the absorption of orally administered polyphenols in the upper gastrointestinal tract is relatively low; a significant portion of polyphenols accumulate in the colon, where they may alter the composition of gut microbiota by exhibiting prebiotic effect and by encouraging the growth of helpful bacteria (Kawabata et al., 2019). The fact that polyphenols have a very low bioavailability in their natural state and are catabolized by enzymes in the small intestine is a significant factor in this circumstance. However, a sizeable portion of dietary polyphenols still travels to the colon, depending on their degree of polymerization and glycosylation (Catalkaya et al., 2020). An oral intake of resveratrol and epigallocatechin-3-gallate (282 and 80 mg/day, respectively) for 12 weeks significantly reduced fecal abundance of Bacteroidetes and tended to reduce

Faecalibacterium prausnitzii in overweight men compared to those taking a placebo, according to a randomized, double-blind, placebo-controlled human trial (Most et al., 2017).

Consumption of resveratrol (0.025% w/w in diet) inhibited the microbiota dysbiosis induced by dextran sodium sulfate (DSS) in colitic mice by significantly elevating the fecal abundance of *Bifidobacterium* and lowering the abundance of *Dorea*, *Sutterella*, and *Bilophila* (Li et al., 2020a). Apart from the pure polyphenols, administration of polyphenol-rich foods/extracts also altered the composition of gut microbiota. Intake of green tea polyphenol extracts for 18 weeks in canines inhibited the abundance of *Bacteroidetes* and *Fusobacteria*, and increased the *Firmicutes* (Li et al., 2020b). Similarly, in a recent study, administration of wild blueberry polyphenolic extract and a fraction isolated from the blueberries (including oligomeric proanthocyanidins with a degree of polymerization less than four phenolic acids and favonols) to high-fat high-sucrose diet-induced obese mice favored the growth of polyphenol degrading bacteria *Adlercreutzia equolifaciens*, suggesting that inclusion of these bacteria in the metabolism of polyphenols may contribute to the amelioration of metabolic disturbances in obesity and diabetes by producing bioactive molecules involved in these processes (Rodríguez-Daza et al., 2020).

1.1.6. Dietary Habits, Gut Microbiota, and their Metabolites

Everybody's diet is distinguished by a particular combination of micro and macronutrients that are continuously and in various amounts fed to our intestinal ecology. Investigating how dietary habits affect the gut flora is therefore interesting. **Table 2** displays the reported alterations in the composition of the gut microbiota in response to various dietary patterns.

Table 2. Effect of different types of diet on commensal bacterial species (adapted by Rinninella et al., 2019)

Vegan/Vegetarian Diet	Gluten-free Diet	Ketogenic Diet	Low Fodmap Diet	Western Diet	Mediterranean Diet
↓ *Bifidobacteria* ↑ *Clostridium clostridioforme* ↑*Fecalibacterium prausnitzii* ↓ *Clostridium cluster XIV* ↑ *Klebsiella penumoniae* ↓ *Bilophila* ↑ *Bacteroides/Prevotella* ↑ *Bacteroides thetaiomicron* ↑ *Bacteroidetes*	↑ *Bifidobacteria* ↓ *Corobacteriaceae* ↓ *Veillonellaceae* ↓ *Ruminococcus bromii* ↓ *Roseburia* ↑ *Victivallaceae* ↑ *Clostridiaceae* ↓ *Lactobacillus* ↓ *Clostridium lituseburense* ↓ *F. prausnitzii* ↑ *Enterobacteria (E. coli)*	↓ *Bifidobacteria* ↓ *Eubacterium rectale* ↓ *Dialister* ↓ *Firmicutes* ↑ *Enterobacteria (E. coli)* ↑ *Desulfovibrio spp.* ↑ *Parabacteroides* ↑ *Bacteroidetes* ↑ *Akkermansia*	↓ *Bifidobacteria* ↓ *Ruminococcus gnavus* ↓ *Clostridium* ↓ *F. Prausnitzii* ↓ *Akkermansia*	↓ *Bifidobacteria* ↑ *Ruminococcus torques* ↓ *Roseburia* ↓ *E. rectale* ↓ *R. bromii* ↓ *Lactobacillus* ↑ *Enterobacteria* ↑ *Bilophila* ↑ *Alistipes* ↓ *Prevotella* ↑ *Bacteroides* ↑ *Akkermansia*	↑ *Bifidobacteria* ↑ *Lactobacillus* ↓ *Clostridium* ↑ *Lachnospiraceae* ↓ *Enterobacteria* ↑ *Bacteroidetes*

1.1.6.1. Vegan and Vegetarian Diets

In contrast to vegans, who additionally abstain from ingesting foods derived from animals, such as eggs, milk and dairy products, honey, the term "vegetarian" refers to all people who eliminate meat and fish from their diet. Studies that compare the intestinal microbiota of omnivores, vegetarians, and vegans have given themselves the purpose of doing so. The microbiota of vegetarians and vegans appears to be different from that of omnivores in that it has higher ratios of *Bacteroides/Prevotella*, higher concentrations of *Bacteroides thetaiotaomicron*, *Clostridium clostridioforme*, *Faecalibacterium prausnitzii*, and *Klebsiella pneumoniae*, and lower concentrations of *Clostridium cluster XIVa* and *Bilophila wadsworthia* (Ruengsomwong et al., 2016). Vegetarians and vegans had lower levels of *Bifidobacterium* and *Bacteroides*, according to another study (Zimmer et al., 2012). However, there was no distinction between vegans and omnivores in terms of the amount of SCFA in stool or the amount of methane exhaled through the breath. According to this finding, diets devoid of animal products alter the microbiota's composition but have no impact on the levels of SCFA or methane. According to the research by Losasso et al. (2018), vegan and vegetarian diets undoubtedly affect the intestinal microbiota, but they do not allow for inferences about its composition.

In fact, the methods used to identify the microbiota, the size of the sample being studied, and variables like geographic origin, age, sex, and body mass, which have a substantial impact on the microbiota, must all be taken into account when interpreting the results (Wong et al., 2018). Along with the

findings, it is important to consider the high levels of polyphenols found in plant-based foods, which are abundant in vegetarian and vegan diets, as well as their effects on the microbiota. In fact, these elements encourage the development of healthy bacteria like *Bifidobacterium* and *Lactobacillus*. However, further research is required to fully understand the intricate mechanisms and interactions between gut microbiota and vegan / vegetarian diets.

1.1.6.2. *Gluten-Free Diet (GFD)*

Celiac disease (CD) is a chronic autoimmune disease that causes an immune reaction of the body in response to the introduction of gluten in the diet. If the immune response is not identified and treated, it results in small intestinal inflammation, which impairs nutrition absorption and puts a person's health at risk. The only way for celiac patients to move forward is with a gluten-free diet, which enables the restoration of normal intestinal mucosa (McAllister et al., 2018; Newnham et al., 2017). A drop in helpful bacteria like *Bifidobacterium, Clostridium lituseburense*, and *Faecalibacterium prausnitzii* and a rise in opportunistic bacteria like *Enterobacteriaceae* and *Escherichia coli* have been observed in healthy patients who have been on a gluten-free diet (GFD) for more than a month. Reduced production of SCFA and, thus, diminished benefits on the host's metabolism and immunity result from the decline in helpful bacteria like *Bifidobacterium* and *Lactobacillus*. Additionally, a study by Bonder et al. (2016) demonstrated that GFD determines changes, particularly in the *Veillonellaceae* family, whose abundance declines noticeably after GFD. This diet also determined a decrease in *Ruminicoccus bromii* and *Roseburia* in the feces and an increase in *Victivallaceae*, *Clostridiaceae,* and *Coriobacteriaceae*. Although GFD over the long term in celiac subjects determines an improvement in the health of the intestine and consequently in the symptoms of celiac disease, an imbalance in the gut microbiota may result by affecting the long-term homeostasis of harmful species like *Enterococcus, Staphylococcus, Salmonella, Shigella*, and *Klebsiella* (Bonder et al., 2016).

1.1.6.3. High-Glucose or -Fructose Diets

Consuming too much sugar has been associated to obesity, metabolic disorders such type II diabetes mellitus, NAFLD (non-alcoholic fatty liver disease), and cardiovascular disease. Eating habits today are particularly rich in sugar (Stanhope et al., 2016) A diet high in glucose or fructose was shown to reduce microbial diversity in a mouse study by Do et al. (2017) compared to the control group (mice fed a normal diet), with fewer *Bacteroidetes* abundance and more *Proteobacteria* abundance as well as an increase in *Desulfovibrio vulgaris* species. There was a notable rise in intestinal permeability in

addition to these alterations in the microbiota's composition. As a result, as compared to controls, mice fed a diet high in glucose or fructose expressed more inflammatory cytokines (TNF-alpha and IL-1beta) in the colon. These findings demonstrate how an increased intake of glucose and/or fructose alters the intestinal microbiota by raising the *Firmicutes/Bacteroidetes* ratio and the amount of *Proteobacteria*, one of the best suppliers of LPS (Rizzatti et al., 2017). Additionally, by modifying the intestinal microbiota, these dietary practices drastically affect intestinal permeability, escalating metabolic endotoxemia, and systemic inflammation (Rinninella et al., 2018).

1.1.6.4. Low FODMAP Diet

All highly fermentable yet poorly absorbed carbohydrates and polyols are referred to as FODMAPs (Gibson et al., 2017b). Patients with IBD and IBS have recently been treated with the FODMAP diet (Catassi et al., 2017; Marsh et al., 2016). In IBS patients, the low-FODMAP diet produced comparable SCFA concentrations but roughly 47% less bacterial abundance than a standard diet (Halmos et al., 2014). The low-FODMAP diet seems to result in a decrease in *Clostridium cluster IV, Propionibacteriaceae, Akkermansia muciniphila*, *Ruminococcus gnavus*, and *Bifidobacteria* when compared to a normal diet (Halmos et al., 2014; McIntosh et al., 2017; Staudacher et al., 2017). In fact, eating a diet low in FODMAPs may lead to a lower intake of prebiotics (FOS and GOS), which may in turn lead to a decrease in good bacteria and the health benefits associated with fermentation by the latter. However, adding probiotics to a diet low in FODMAP may help to restore the intestinal microbiota's balance and bring back *Bifidobacterium* abundance to pre-diet levels (McAllister et al., 2018). However, more research is needed to fully understand the advantages of probiotic supplementation and the long-term effects of the low-FODMAP diet on the gut microbiota.

1.1.6.5. Western Diet

The Western Diet (WD) is the diet followed by many people in developed countries and increasingly in developing countries, associated with economic growth. The WD diet is rich in fats, animal proteins, and refined sugars. According to a study by Martinez Medina et al. (2014), a diet high in fat and sugar leads to gut microbiota dysbiosis in mice due to an increase in *Bacteroides spp*. and *Ruminococcus torques*. Using the microbiota characterization of volunteers from Venezuela, Malawi, and the United States as a starting point, another study (Yatsuneko et al., 2012) revealed that, regardless of the volunteers' personal data, subjects from Malawi and Venezuela had a composition of the microbiota similar to that of the volunteers from the United States, who were instead distinguished by a lower microbial diversity. This study showed that a diet high in animal proteins

decreased levels of *Firmicutes*, which metabolize dietary plant polysaccharides, such as *Roseburia, Eubacterium rectale,* and *Ruminococcus bromii,* and increased levels of bile-tolerant microorganisms such *Alistipes, Bilophila,* and *Bacteroides* (David et al., 2014). Additionally, numerous studies have demonstrated that food additives can exacerbate the detrimental effects of WD on the intestinal microbiota by causing dysbiosis, which in turn has detrimental consequences on the intestinal mucosa and inflammation (Chassaing et al., 2015). WD, which results in gut microbial dysbiosis, may also be linked to obesity and metabolic disorders, according to a number of studies (Zinocker et al., 2018).

1.1.6.6. Mediterranean Diet

The term "Mediterranean diet" (MD) describes the eating patterns of people who live in the Mediterranean region, primarily in Crete, the majority of the rest of Greece, and southern Italy (Willett et al., 1995). MD, which is high in fruits, vegetables, olive oil, nuts, legumes, and whole grains, is believed to have a number of health benefits, including lowering mortality risk and preventing many diseases like cardiovascular disease, diabetes, metabolic syndrome, cognitive impairment ìand depression (Rosato et al., 2019). The benefits of this diet can be attributed to its regular consumption of MUFA and PUFA, polyphenols, and other antioxidants, as well as to its high intake of fiber and carbs with a low glycemic index and to its preference for plant-based proteins over animal ones.

It has been shown that MD determines a greater presence of *Bacteroidetes* in the intestinal microbiota, probably due to a lower intake of animal proteins, and of total *Bifidobacteria* and SCFA, related to the high consumption of foods of plant origin (Garcia-Mantrana et al., 2018). These results were also confirmed by a study by Mitsou et al. (2017) which demonstrated the positive correlations between MD and increased total intestinal bacteria count and in particular the *Bifidobacteria / E. coli* ratio, the relative share of total *Bacteroides, C. albicans* and SCFA, as well as the decrease in *E. coli* levels. All of these findings appear to support the relationship between MD and a well-balanced gut microbiota that exhibits high diversity and richness.

1.1.6.7. Lactose-free diet

Lactose, a disaccharide, is the most predominant carbohydrate in mammalian milk. Disaccharides are only partially absorbed thus lactase transforms milk lactose into glucose and galactose so that it can be used. People who are lactose intolerant have symptoms like diarrhea, abdominal pain, bloating, and flatulence after consuming dairy products that contain lactose because undigested lactose moves from the small intestine to the colon. The colon is likely osmotically loaded with undigested lactose,

gas, and other metabolites from bacterial fermentation, which is the cause of these symptoms (Smith et al., 2022). Bacterial fermentation in the stomach is one possible route of lactose breakdown in non-lactase-persistent people. According to multiple research the relationship between *Actinobacteria*, specifically *Bifidobacterium*, and LCT variations, a gene encoding for the lactase enzyme, is by far the most statistically significant (Blekhman et al., 2015; Goodrich et al., 2016; Bonder et al., 2016; Hughes et al., 2020). This connection shows that *Actinobacteria* and humans have a substantial interaction (Trosvik et al., 2015). According to a study of Qin et al. (2022), those with genetic lactose intolerance who reported consuming dairy products frequently had significantly higher *Bifidobacterium* levels (Wang et al., 2016). In individuals with lactase deficiency, it's likely that consumed lactose becomes available for colonic bacteria to compete for as an energy source. There have been suggestions of a rivalry between *Bifidobacterium* and *Negativibacillus*, which may be influenced by lactose consumption and needs to be investigated in functional studies. The impact of milk consumption on the gut flora has not been the subject of many comprehensive investigations. Recent studies have looked into the potential prebiotic effects of milk oligosaccharides on the gut flora. Charbonneau et al. (2016) showed that bovine milk sialylated oligosaccharides (BMOs) boost weight gain related with the reactivity of Bacteroides fragilis and *Escherichia coli* to BMOs under situations of starvation using "humanized" animal models with the gut microbiota of children. Karav et al. (2016) shown that BMOs released from glycoproteins can mimic the selectivity of human milk oligosaccharides (HMOs) for *Bifidobacterium* strains in the gut microbiota of young children. Boudry et al. (2017) discovered that a diet supplemented with BMO improved intestinal barrier function, increased the variety of cecal and colonic microbiota, and boosted the relative abundance of *Lactobacillus* in a mouse model of food-induced obesity. In people who are lactose intolerant, lactose fermentation by saccharolytic (sugar-digesting) bacteria can cause abdominal discomfort. However, this approach also has advantages. Colon health depends on SCFAs and other fermentation byproducts that release additional calories from normally indigestible carbohydrates. Colonocyte differentiation, glucose and lipid balance, and immunological regulation are all regulated by SCFAs produced by microbial fermentation of lactose, with consequences for homeostasis and gut-brain modulation (Tan et al., 2014; Canfora et al., 2015; Goncalves et al., 2013; Dalile et al., 2019). Overall, some experts claim that consuming trace amounts of foods containing lactose may have "more to gain than to lose" for people who are lactase intolerant (Lukito et al., 2015). To support the use of dairy products, the gut microbiota also adapts. Therefore, even though lactase expression is not regulated by lactose ingestion, regular lactose consumption seems to lower hydrogen excretion in the breath and reduce lactose intolerance symptoms (Misselwitz et al., 2019). Increases in *Bifidobacteria* and/or *Lactobacilli*, which are considered to be beneficial microbiome components, have been observed in

studies conducted both *in vivo* and *in vitro* (Misselwitz et al., 2019). In a comprehensive study of healthy Japanese people, the prevalence of *Bifidobacteria* was found to be positively correlated with dietary intake of dairy products (Kato et al., 2018). It's possible that the fact that this population comprises 90% to 100% LNP (non-persistent lactase) indicates how routinely consuming lactose impacts the microbiota, but it's also likely that the opposite is true.

1.1.6.8. Effect of diet on microbial metabolite production

A healthy metabolism is essential for wellbeing. The development of metabolic illnesses including obesity, diabetes mellitus, dyslipidemia, and osteoporosis, on the other hand, is typically brought on by changed metabolic pathways (Tabatabaei-Malazy et al., 2015). People with metabolic dysregulation may benefit from lifestyle changes, such as dietary adjustments and the adoption of a more active lifestyle, as alternatives to medicinal or surgical therapies (Lee et al., 2021). Due to this, numerous clinical studies have been conducted to determine how dietary changes can affect various metabolic illnesses (Cotillard et al., 2013; Cummings et al., 2016). To date, mass spectrometry (MS)-based metabolomics is considered a useful tool in clinical trials to investigate the effect of dietary interventions in improving metabolic disorders, including the discovery of potential biomarkers of the pathophysiology of these syndromes (Perdomo et al., 2019).

These analytical tools are increasingly being used in dietary interventions linked to human metabolic disorders, particularly in the identification and measurement of small molecular weight compounds, volatile organic compounds (VOCs), and short-chain fatty acids (SCFAs), in human biological fluids like plasma and feces (Wan et al., 2019; Santini et al., 2016; van der Beek et al., 2018; Jasbi et al., 2019). The production of SCFA by the gut microbiota from the fermentation of indigestible components is typically necessary to maintain optimal gut health (Baxter et al., 2019). Changes in diet-mediated processes and physiological processes in the gut affect SCFA synthesis (Rios-Covian et al., 2016). Therefore, a rigorous evaluation of SCFA levels may be essential to comprehending the conditions of health and disease. It is well known that humans lack the necessary enzymes to digest the majority of dietary fibers, and that the colonic/intestinal microbiota ferments non-digestible carbohydrates in the large intestine (Rios-Covian et al., 2016; Den Besten et al., 2013). As a result of dietary fiber fermentation, SCFA become the major class of metabolites produced by the gut microbial community (Nicholson et al., 2012). Acetate (C2), Propionate (C3), and Butyrate (C4) have been reported to be the most numerous SCFA, making up 90–95 percent of the SCFA found in the colon. SCFA are molecules with one to six carbons (Rios-Covian et al., 2016). Previous research has shown that a variety of factors, such as dietary intake patterns, antibiotic use, and microbial populations, have an impact on SCFA synthesis (Rios-Covian et al., 2016; Velikonja et al., 2019; Lee

et al., 2020; Kasote et al., 2018). While amino acids from the breakdown of proteins, such as Valine, Leucine, and Isoleucine, are also involved in the formation of branched SCFAs such Isobutyrate, Isovalerate, and 2-Methylbutyrate, carbohydrates are the primary source of SCFA generation (Rios-Covian et al., 2016). Epidemiological studies have demonstrated that the gut microbiome can use SCFAs as an energy source for host cells and take part in host signaling pathways through microbial metabolites (Rios-Covian et al., 2016). By integrating glucose and lipid metabolism, SCFAs can also affect the host's physiology and perhaps control the onset of metabolic syndrome (He et al., 2020; den Besten et al., 2015; Scoville et al., 2019). Additionally, SCFAs are essential for preserving the integrity of the intestinal barrier because they provide energy to the intestinal epithelial cells, which may promote the production of mucin and halt the progression of intestinal disorders (Liu et al., 2021). Butyric acids have been shown to increase the integrity of tight junctions and prevent bacterial adherence (Jung et al., 2022).

Based on diet-induced changes in metabolic and anthropometric parameters, numerous studies have shown the favorable benefits of dietary components on the prevention of the risk of developing metabolic abnormalities (der Beek et al., 2018; Canfora et al., 2017; Salazar et al., 2015; Fava et al., 2013; Larsen et al., 2013). Small-molecule metabolite biomarkers, in particular SCFAs, have been considered crucial markers of the metabolic alterations behind physiological changes following nutritional intervention. In general, dietary fibre (such as resistant starch from barley) or carbohydrate-based diets were associated with increased bacterial abundances and raised SCFA levels (Salonen et al., 2014; Bouter et al., 2018; Canfora et al., 2017; Hald et al., 2016). In a study of Velikonja et al. (2019), a group of people with MetS or at high risk of acquiring it received either bread with additional beta-glucans as a test diet or bread without it as a control. Following the dietary intervention, there were noticeably altered levels of SCFA in the feces, with the control group experiencing an increase in propionic acid (Velikonja et al., 2019). In other instances, despite increasing levels of some bacteria found, therapies with Sodium butyrate and inulin-type fructans have been seen to lower SCFA content (Bouter et al., 2018; Salazar et al., 2015). For instance, Salazar et al. found that obese people who drank inulin-type fructans had considerably lower levels of Acetic, Propionic, and Caproic acid than those who consumed maltodextrin as a control (Salazar et al., 2015).

1.1.7. Perturbation of gut microbiota following the use of antibiotics

Drugs are now recognized as having a significant impact on the composition of the human gut microbiota (Maier et al., 2018; Falony et al., 2016; Rothschild et al., 2018; Zimmermann et al., 2021). It is recognized that antibiotics designed to have broad-spectrum activity also directly alter the gut microbiota, even if the importance of non-antibiotic medications has, until recently, been

underappreciated (Maier et al., 2018; Zimmermann et al., 2021; Vich Vila, 2020). As a result, they lead to a variety of gastrointestinal side effects, such as *Clostridioides difficile* infections (Kuhn et al., 2016). Recently, there has been more focus on the adverse effects of antibiotics on the gut flora. Various allergy, metabolic, immune, and inflammatory dysfunctions have been linked in vivo studies between antibiotic-induced long-term microbiome modifications and these dysfunctions (Cho et al., 2012; Cox et al., 2014; Ruiz et al., 2017; Korpela et al., 2016; Parker et al., 2017). However, technical difficulties hinder routine testing of antibiotic susceptibility of anaerobic bacteria and available data on bacterial susceptibility to antibiotics offer minimal resolution on human gut bacteria. Different antibiotic class responses have been observed (Maier et al., 2021). Quinolones, for instance, behaved in accordance with their generation. Only a few bacterial species were resistant to first-generation variants; however, second- and third-generation quinolones exhibited broader activity, and fourth-generation variants (created to improve activity against anaerobes) inhibited nearly all species tested (Maier et al., 2021). For β-lactams, resistance was phylogenetically inhomogeneous, which was corroborated by further data collected for *Bacteroides* strains and species (Maier et al., 2021). Within the genus *Bacteroides*, phylogenetic relatedness and susceptibility to -lactams were shown to be ambiguous (Maier et al., 2021). This implies that β-lactam resistance mechanisms are strain-specific and horizontally transferred. Macrolides showed a strong effect on intestinal commensals and inhibited all tested microorganisms except *C. difficile*, which was resistant to macrolides and clindamycin (Maier et al., 2021), consistent with the known risk of *C. difficile* infection after treatment with macrolides or clindamycin (Slimings et al., 2014). The pig could be viewed as a potent translational model of the gut microbiota for elucidating the effects of antibiotics on the gut microbiota and for developing new treatments and prevention strategies. Indeed, the gastrointestinal anatomy and physiology of pigs and humans are remarkably similar (Rose et al., 2022).

1.1.8. Gut microbiota of pigs

Under natural conditions, more than 90% of the bacteria present in the colon of humans and the adult pig fall into one of two phyla: *Firmicutes* or *Bacteroidetes* (Gao et al., 2018; Isaacson et al., 2012; Looft et al., 2012; Lamendella et al., 2011; Guo et al., 2008; Crespo-Piazuelo et al., 2019). Although there is slight variation in bacterial genera and species due to species specificity, the shared bacterial physiology and metabolism within these phyla solidifies the adult pig as a viable model of the healthy human colonic microbiota. When comparing the microbiota of newborn pigs and human infants' big intestines, it is easier to see how different the two species are from one another. Adult and neonatal pigs have much more *Actinobacteria* in their large intestine contents and feces than do adults or neonates (Lamendella et al., 2011, Wang et al., 2015). In fact, *Actinobacteria* predominate in both

breastfeeding and formula-fed infants' big intestines. Even though the same two phyla predominate in the large intestines of adults and mature pigs, comparing bacterial genera isolated from these phyla helps to further understand interspecific diversity. The most prevalent genus of the family *Bacteroidetes* in the human gut is *Bacteroides*, while *Prevotella* predominates in the gut of pigs (Roura et al., 2016). *Prevotella* makes up much to 30% of the pig colonic microbiota at the age of 10 weeks. When the pig reaches 22 weeks of age, however, the relative number of *Prevotella* species drops to 4% and the relative number of *Anaerobacter sp.* belonging to the phylum *Firmicutes* increases (Lamendella et al., 2011; Kim et al., 2011). The first four months of life in humans show a consistent decline in *Bacteroidetes* and a rise in *Firmicutes* (Vaiserman et al., 2020). Therefore, even though there are some specific bacterial genera and species that differ between the guts of pigs and humans, it is likely that bacterial phyla that are shared cause analogous physiological processes and create equivalent symbiotic interactions. The existence of certain microbes that are peculiar to pigs is one of the most evident variations between the microbiotas of humans and pigs. In fact, both sow-reared and formula-fed piglets exhibit greater gut microbial diversity than human neonates (Wang et al., 2015). *Fusobacterium* is found in small amounts in the feces of neonatal pigs but not in breastfed neonates (Wang et al., 2015). Pig gut has far more lactobacilli, spirochetes, and streptococci than human intestine at any age (Roura et al., 2016; Lamendella et al., 2011). *Proteobacterium* make up a significant portion of the microbiota in the ileum of pigs, while not being present in the human ileum (Isaacson et al., 2012). However, significant amounts of *Proteobacteria* have been found in the feces of breastfed children (Wang et al., 2015). Similar changes in the gut microbiota are observed in adult pigs and humans in response to environmental stresses and antibiotics (Isaacson et al., 2012; Looft et al., 2012; Dethlefsen et al., 2008; Lamendella et al., 2011; Guo et al., 2008; Borewicz et al., 2015). This implies that the pig is an effective model for pathological gastrointestinal conditions, such as IBD and antibiotic-induced dysbiosis.

1.2. Probiotics and prebiotics

In the recent years, prebiotics and probiotics have drawn increasing attention from the scientific, medical, and public sectors. Public awareness of microorganisms has grown as a result of advertising on the topic of microbiome research, which has also helped people recognize the positive impact that microbes have on human health. Because of this increased understanding, probiotics and prebiotics are becoming more widely accepted (Chin-Lee et al., 2014), with the probiotic sector rising by 7% yearly (Jackson et al., 2019) and prebiotics anticipated to rise by 12.7% over the next eight years (Mano et al., 2018). Although prebiotics and probiotics have a favorable reputation among consumers, their definition, beneficial effects on health, and mode of action are still unclear (Chin-

Lee et al., 2014; Viana et al., 2008). New probiotic and prebiotic options are being investigated, and more in-depth knowledge about their interactions with the microbiota and the host is now being made available thanks to technological advancements in data gathering and analytical tools.

1.2.1. Probiotics – novel species and health targets

Lactobacilli, bifidobacteria, and other lactic acid bacteria (LAB), mostly isolated from fermented dairy products and fecal microbiota, have been employed frequently as probiotics. A novel spectrum of microbes from human microbiomes have been isolated and characterized as possible next-generation probiotics thanks to advancements in complete genome sequencing and cultivation techniques (O'Toole et al., 2017). There is rising interest in the probiotic potential of a number of bacteria that have been isolated from the human intestine, including *Roseburia intestinalis*, *Faecalibacterium prausnitzii, Eubacterium spp., Bacteroides spp.*, and *Akkermansia muciniphila* (O'Toole et al., 2017; Brodman et al., 2017). These candidates represent a large chunk of the currently cultivable human gut microbiome and offer physiological solutions that are not always obtained directly from bifidobacteria or lactobacilli, such as the production of butyrate, propionate or other bioactives (Blaak et al., 2020). There are restrictions on using these species as industrially viable probiotics, including as their requirement for anaerobic environments and rich growth media. In addition to the human gut microbiome, the female urogenital tract, mouth cavity, nasopharyngeal tract, and skin are all excellent sources of novel potential probiotic strains (Maguire et al., 2017; George et al., 2016; Cribby et al., 2008). Researchers are looking into possible therapies to return microbial populations to disease states that are related to the species or genera connected with the health of these body parts. Examples include the use of *Lactobacillus crispatus* for vaginal dysbiosis (Reid et al., 2012) and *Staphyloccocus hominis* for eczema and atopic dermatitis (Nakatsuji et al., 2017). Consuming fermented foods has been linked to major health benefits, such as a decreased risk of diabetes 2 and cardiovascular disease and a metabolic profile that is presumably advantageous. Fermented foods are a rich source of possibly probiotic LAB strains (Taylor et al., 2020). Fermented and unfermented foods potential sources of future probiotics are fruits, vegetables, grains, dairy, meat and fish products and honey, as well as environmental sources such as soil (Zielìnska et al., 2018). In addition to probiotics, postbiotics - microbial fragments and metabolites (Aguillar-Toalà et al., 2018) - have been shown to share many mechanisms of live probiotics. Most likely, some promising new intestinal isolates will also be marketed as postbiotics, such as pasteurized *A. muciniphila* or bioactive proteins from this species which have shown beneficial effects (Plovier et al., 2017).

1.2.2. Prebiotics

1.2.2.1. Evolution of the term prebiotic

Modulating the microbiota to enhance human health is a developing concept that is a component of a thorough and all-encompassing approach to lifestyle wellness. Mucosal and skin surfaces are home to rich and diverse microbial communities that serve as targets for methods to maintain or promote health or treat disease. Now, dietary or non-dietary interventions are able to change the makeup and metabolizing signatures of these microbial populations (David et al., 2014; Cani et al., 2016). Prebiotics are a group of chemicals that have been known for more than 20 years to be able to influence the host's microbiota for the host's advantage (Gibson et al., 1995). At the time, fructans (fructo-oligosaccharides or FOS and inulin) and galactans (galacto-oligosaccharides or GOS) fell into this category, with effects acting through enrichment of *Lactobacillus* and/or *Bifidobacterium spp.* FOS and GOS currently dominate the prebiotic category, as evidenced by numerous studies on their prebiotic effects. The concept of a prebiotic has evolved over time, in part because of improvements in microbiome research methods (such high throughput sequencing), which have increased our understanding of the microbiota's makeup and allowed us to find more compounds that affect colonization. With these developments, it has become clear that prebiotics affect a greater variety of beneficial bacteria and that they may also be effective in extraintestinal locations, either directly or indirectly (Collins et al., 2016). Prebiotics are non-digestible food ingredients that have a positive impact on the host by specifically stimulating the growth and/or activity of one or a small number of bacteria already present in the colon. Although dietary oligosaccharides have long been used to provide health benefits, especially in Asia, the term prebiotic was first used in 1995. (Gibson et al., 1995). Prebiotics work to alter the microbiota found in both people and animals in order to enhance health. Prebiotics are non-viable substrates that provide nutrients for host-hosted beneficial bacteria, such as given probiotic strains and native (resident) microorganisms, as opposed to probiotics, which use living microbes. As a result, prebiotics are different from the majority of dietary fibers, such as pectins, cellulose, and xylans, which encourage the growth of a wide range of intestinal microbes. A prebiotic should not cause a broad metabolic response, but rather one that is focused on the ecosystem's native, health-promoting bacteria. Most of the first prebiotics evaluated in humans and used commercially have been shown to specifically stimulate *Lactobacillus* and *Bifidobacterium*, but not pathogens such as some members of the *Clostridia* class and *Escherichia coli* (Depeint et al., 2008; Costabile et al., 2010; Roberfroid et al., 2010). This method established a connection between probiotics and prebiotics because these genera are frequently employed as probiotics. Prebiotics are now understood to be "selectively fermented nutrients that enable specific changes in either the composition or activity of the gastrointestinal microflora that confer benefits to the well-being and health of the host," according to a definition revision made in 2004. (Gibson et al., 2004). This

definition stipulated that a prebiotic must meet three requirements: it must be able to withstand the host's digestion (such as gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption); it must be fermented by gut microorganisms; and it must specifically promote the growth and/or activity of gut bacteria linked to health and wellbeing. It followed that tests to show the effects of prebiotics had to be carried out on the target host. In the absence of research showing effects on host health, in vitro assessments to identify pathways or processes would not be able to prove prebiotic status. A technical meeting to update the definition of a prebiotic was organized by the Food and Agriculture Organization of the United Nations (FAO) in 2008. By redefining prebiotics as "a non-viable dietary component that offers a health benefit to the host associated with regulation of the microbiota," this group hoped to improve the definition of the term (Pineiro et al., 2008). In this instance, the definition was criticized for not excluding antibiotics because selective fermentation was removed as a requirement. Two years later, Gibson et al. (2010) outlined the more specific definition of "dietary prebiotics," which they defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thereby conferring health benefits on the host."

In 2015, Bindels et al. proposed to remove specificity requirements based on reports showing that prebiotics enrich multiple taxa, rather than particular species (Dewulf et al., 2013). This proposal led to another definition of a prebiotic as 'a non-digestible compound that, through its metabolisation by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thereby conferring a beneficial physiological effect on the host' (Bindels et al., 2015). With the removal of the need for selective fermentation, this definition restricted prebiotics to interactions with the gut microbiota (avoiding extraintestinal locations like the vagina and skin). This group believes that the key to the prebiotic idea is selectivity with regard to microbial fermentation. However, it is crucial that this description emphasizes how prebiotics affect the microbiota in a functional manner. A consensus definition is now required due to the above mentioned proposed definitions as well as others (Hutkins et al., 2016). Gibson et al. (2017) therefore suggested the following definition of a prebiotic: a substrate utilised specifically by host bacteria that delivers a health advantage.

1.2.2.2. Prebiotic effect and selectivity

The word "selectively" was used in early definitions of the term prebiotic to mean principally lactobacilli and bifidobacteria. Bifidogenesis, the specialized stimulation of bifidobacteria, was seen as a prebiotic effect. Early studies on the ecology of the bacteria in the gut relied heavily on culture techniques, which are now known to be insufficient for revealing the complexity of microbial alterations brought about by prebiotics. Understanding that certain bacterial genera may utilize

particular prebiotic materials through fermentation and other metabolic pathways has been made possible by molecular approaches, which have now uncovered a wider range of gut microbial community members. Depending on the host and ecology under consideration, these bacteria may differ. Therefore, it is widely acknowledged that the effects of prebiotics likely extend beyond bifidobacteria and lactobacilli, but the spectrum of microorganisms involved must be restricted to meet the prebiotic's selectivity criterion. To this purpose, bifidobacteria responded to the use of prebiotics in two human trials employing high-throughput sequencing (Dewulf et al., 2013; Vandeputte et al., 2017). *Faecalibacterium prausnitzii*, for example, also increased in abundance in a study (Dewulf et al., 2013), while *Anaerostipes spp*. grew further in a different study, but *Bilophila spp*. dropped (Vandeputte et al., 2017). High throughput sequencing was employed in both investigations to verify the selectivity of prebiotic fermentation. A selective effect may affect numerous microbial groups, but not all of them; selectivity is not always associated with effects on just one microbial group. A prebiotic must produce a net health benefit in addition to having a selective impact on bacteria. The underlying ideas are that the metabolites generated and the microbes affected are both thought to be advantageous and connected to a certain component of health. It's challenging to visualize each situation. Is a food a prebiotic, for instance, if consuming it stimulates butyrate synthesis in microorganisms? It is acknowledged that certain molecules, including short-chain fatty acids (SCFA) like acetate, propionate, and butyrate, are mechanistically associated to certain health effects (Canfora et al., 2016: Koh et al., 2016). It would be considered a "prebiotic effect" if the effect results in a demonstrable improvement in the host's health when compared to a control. Experiments would need to prove that the product is used selectively, in this case by showing that a specific spectrum of butyrate-producing microbes develop as a result of the product, in order to confirm that the product is prebiotic in and of itself. Alternately, the product can release compounds that in turn encourage the formation of butyrate by more bacteria, stimulating the growth of other microbiota individuals. This occurrence might have a "cross-feeding effect." As this overall health effect was caused by the spread of certain microbes, the overall outcome is still selective. However, one cannot refer to a prebiotic if pathogenic microbes are involved in the production of butyrate and a bad outcome for the host ensues. The function and makeup of the intestinal microbiota involved must be determined in light of this differentiation. Similar to probiotics, prebiotics must not cause difficulties with gas distension after consumption in the human gut microbiota; as a result, their fermentation must be selective and preferably include non-gas-forming species (such as *Clostridium*). This point categorically shows that selective metabolism is necessary. Bifidobacteria and lactobacilli in particular don't create gas during their metabolism (Roberfroid et al., 2010).

Additionally, it is emphasized that these effects on human health need to be studied in mixed microbial communities that incorporate the complete microbiota of interest (i.e. *in vivo*). It is insufficient to draw conclusions regarding prebiotic effects from pure trials or in coculture. Similar to this, judgments about prebiotic activity must be drawn from an analysis of the complete microbial variety rather than just an increase in the number of, say, bifidobacteria or gut lactobacilli. The best methods must be used, particularly in light of the fact that the study of the microbiome has considerably profited from molecular-based technology advancements. High-throughput sequencing methods, such as metagenomics, which show quantitative changes in the microbiota, are among these techniques. Similar to this, metabonomic analyses using appropriate biological materials, such as NMR or mass spectrometry, can uncover metabolic responses to prebiotics and assist in determining the simultaneous functionality of the microbiota.

1.2.2.3. Substrates that are prebiotics

Numerous fermentable carbohydrates have been shown to have a prebiotic effect, but the non-digestible oligosaccharides fructans and galactans are the food prebiotics whose health advantages in people have been most thoroughly proven (Rastall et al., 2015). Bifidobacteria metabolize these oligosaccharides preferentially (Roberfroid et al., 2010). The enzymes galactosidase and fructoanosidase, which are both common in bifidobacteria, may easily breakdown the bonds in FOS and GOS, a phenomenon that can be explained by the structure-function relationship. Additionally, this genus appears to selectively metabolize oligosaccharides with a degree of polymerization (DP) between 4 and 30. (Rastall et al., 2010; Sarbini et al., 2011). The selectivity of prebiotics at target sites and their capacity to function in a competitive environment in mixed-culture ecosystems like the human gut depend on the presence of the proper transport mechanism to capture and transport these substrates into the microbial cytoplasm (Goh et al., 2015). Prebiotics are not substances that alter the composition of the microbiota through processes other than those involving selective use by host bacteria. These substances, which do not support growth but may affect the microbiota and metabolism upon ingestion, include antibiotics, minerals, vitamins, and bacteriophages. Prebiotic status is possible for some soluble fermentable fibers (Delcour et al., 2016) as well as other dietary fibers if the host microbiota uses them selectively and they benefit human health. The fact that a dietary fiber may be prebiotic in one host but not in another complicates the definition of fibers as prebiotics. For example, cellulose may be considered a prebiotic in ruminants but not in humans, as the latter's gut microbiota poorly utilises glucose-bound β(1→4) polysaccharides (Ben David et al., 2015). Furthermore, the target site may also determine whether a substrate qualifies as a prebiotic.

For instance, xylitol may be considered a prebiotic in the oral cavity but not in other areas (Roberfroid et al., 2010; Gibson et al., 2004). The oligosaccharides contained in human milk are among the first class of chemicals to be recognized for their capacity to affect gastrointestinal health. Human milk oligosaccharides (HMOs) are crucial for the growth of the infant's immune, metabolic, and intestinal flora, all of which have an impact on long-term health (Garrido et al., 2015; Oozer et al., 2013). Breast milk consumption clearly increases the percentage of *Bifidobacteraceae* and *Bacteroidaceae* that consume HMOs (De Leoz et al., 2015). Only one *Bifidobacterium* species, *Bifidobacterium longum subsp. infantis (B. infantis*), has developed machinery specifically designed to break down the entire HMO repertory. Other *Bifidobacterium* species that are common in adults, primarily *B. longum subsp. longum, B. adolescentis*, and *B. lactis*, lack a number of the enzymes necessary to successfully use HMOs in this manner (De Leoz et al., 2015; Rockova et al., 2012). HMOs have metabolism-independent methods of action in the developing gut and may indirectly affect the makeup of the gut microbiota by modifying immunological responses (He et al., 2016). A controlled human investigation proving the selective growth of bifidobacteria with subsequent health benefits is also required to prove their classification as prebiotics. However, the use of such compounds for in vivo studies is limited to a few reports. In one study, a chemically synthesised compound, 2′fucosyllactose (2′FL), equivalent to the naturally occurring 2′FL in HMO, was added to infant formula along with GOS. Although safe for infants, treatment with 2′FL provided no net difference in weight, length, head circumference and other measures compared to human milk over a 4-month period (Marriage et al., 2015). In another study of the same group, infants fed formula with 2′FL plus GOS had similar immune responses to breastfed infants, as both groups had lower levels of inflammatory cytokines than infants fed formula plus GOS (Goehring et al., 2016). However, no effects on the microbiota were reported in this study. In a third study, 2′FL and another synthesised HMO, lactoneotetraose, were administered to adults (Elison et al., 2016). The treatments were well tolerated and resulted in increased abundance of Bifidobacterium spp. Overall, these studies provide an incomplete assessment of the prebiotic properties of these synthesised versions of HMOs. Although 2′FL is utilised by *B. infantis* and some strains of *B. longum subsp. longum* and *B. breve* (Elison et al., 2016; Underwood et al., 2015), the ecological context (i.e. infants versus adults) could determine whether these HMOs are indeed prebiotic. Furthermore, structural equivalence with specific HMOs does not imply functional equivalence with the constellation of HMOs in milk (Carbonneau et al., 2016). Therefore, for now, it is acceptable to state that some HMOs are prebiotic candidates.

Plant polyphenols are a class of substances that may also meet the requirements for prebiotics, albeit much more research in the target host is necessary. According to estimates, 90 95 % of dietary polyphenols do not absorb in the small intestine and end up in the colon (Clifford et al., 2004), where

the colonic microbiota extensively transforms them. More and more evidence suggest that rather than the parent substances, the health advantages of consuming polyphenols depend on how they are used by microbes and the metabolites they produce (Duenas et al., 2015). This information broadens the definition of prebiotics to include other non-digestible oligosaccharides besides FOS and GOS. However, there is less evidence for these newly discovered prebiotics than for fructans and galactans (Roberfroid et al., 2010), and further research examining their potential health advantages is required to establish their classification as prebiotics.

Prebiotics will probably be isolated from new sources in the future as focus to sustainability, cost, and scale emerges (Mano et al., 2018). The food chain generates 1.3 billion tonnes of food waste annually, which is a rich and sustainable source of organic bioactive compounds. Many secondary streams from the processing of fruits, vegetables, and grains contain potential prebiotics, including pectin from orange peel and arabinoxylans from waste from distilleries and breweries (Gomez et al., 2014; Monteagudo-Mera et al., 2018). Future prebiotic molecules may potentially undergo chemical or structural alterations to alter their usefulness. These processes include sonication, high pressure, acid, enzyme, and oxidation. Additionally, novel prebiotic combinations in optimized mixtures may provide the opportunity to develop new benefit profiles (Lam et al., 2019). The use of prebiotics to affect the microbiomes of other hosts, such as the female urogenital tract, oral cavity, and skin, is also gaining popularity. For instance, prebiotic glucomannan hydrolysates have been demonstrated to alter the skin microbiome and lessen acne when applied topically (Al Ghazzewi et al., 2014). For the treatment or prevention of colorectal cancer and ulcerative colitis, there is also interest in a more focused administration of prebiotics in the distal colon. The delivery of intact prebiotics to the distal colon and the selective stimulation of carbohydrate-metabolizing genera may be made possible by a combination of prebiotics with varying chain lengths or by particular delivery techniques. This may reduce local proteolysis and the ensuing production of undesirable metabolites. Beyond merely promoting microbial growth, this modification of the colonic microbial metabolome toward a healthier profile is expected to become a major target for prebiotics (Enam et al., 2019). Table 3 shows old and novel prebiotics according to the last definition.

Table 3. Old and novel prebiotics

PREBIOTIC	**MICROBIAL GROUP**	**HEALTH BENEFIT**	**REFERENCES**
Resistant starch	*Ruminococcus* *Eubacterium*	Increase of concentration of SCFAs and insulin sensitivity	Yang et al., 2017 Lokyer et al., 2017

Polyphenols (proposed as)	*Eubacterium* *Lactobacillus*	Improvement of glucose tolerance and cognitive function; Decrease of hepatic steatotis	Alves-Santos et al., 2020
SCFAs	*Clostridium group IV* *Akkermansia* *Bifidobacteriaceae*	Acetate: improvement of immunological functions, reduction of concentrations of lipids in the blood Propionate: anti-inflammatory properties, cholesterol synthesis inhibitor Butyrate: energetic substrates for host cells, induction of differentiation on staminal crypts	Rios-Covian et al., 2016 He et al., 2020 Liu et al., 2021 Gibson et al., 2017
GOS FOS XOS	*Bifidobacteriaceae* *Lactobacillaceae* *Collinsella* *Akkermansia*	Increase peak expiratory flow and reduce systemic production of type 2 Thelper cytokines after allergen challenge in adults with allergic asthma	Halnes et al., 2017 Carlson et al., 2018
Beta Glucans	*Collinsella* *Akkermansia*	Cholesterol-lowering properties of beta-glucans; many propionate-producing bacteria have a preference to fermenting various type of beta-glucans (*Bacteroides, Prevotella, Clostridium*)	Carlson et al., 2018 Lam et al., 2018

1.3. Gut *in vitro* models

Numerous studies have shown that the host cannot properly metabolize or absorb numerous dietary components, such as polyols or phenolic chemicals, unless the gut microbiota has first altered them (Possemiers et al. 2011). The balance between advantageous, commensal, and opportunistic species in the intestinal microbiota, known as eubiosis, is particularly crucial. Loss of this balance encourages the onset of disorders such inflammatory bowel disease (IBD), malabsorption, and metabolic syndromes (Qin et al. 2012), as well as other conditions (Frank et al. 2007). Understanding the positive effects of the microbiota balance on the host's health depends largely on how the food affects the eubiosis of the microbiota (David et al., 2014). *In vivo* research on the human gut is constrained for ethical reasons and is only permitted in cases of diseases or pharmaceutical trials, making this form of study challenging. As a result, during the past 35 years, researchers from all over the world have created a variety of intestinal fermentation *in vitro* models to simulate how humans digest food or each of its component parts, as well as the function of the accompanying microbiota (Guerra et al. 2012; Venema and Van den Abbeele 2013; Koutsos et al. 2017). These tools facilitate us to investigate the effects of various components, including infections, bioactive agents, dietary molecules, pharmaceuticals, and hazardous substances, on the intestinal microbiota. To research the human intestinal microbiota's metabolism and changes through time, *in vitro* models are used to

cultivate it in the most accurate manner under tightly regulated environmental circumstances. The key drawback of these models is that they do not include immunological and epithelial cells, which are essential in host-microbe interactions. Looking to highlight some applications of the *in vitro* gut fermentation models, in **Table 4** are reported some studies applied to the described systems.

Table 4. Recent applications of intestinal *in vitro* models

MODEL	Most representative applied studies	References
Batch fermentation model	Potential of edible insects to modulate the human gut microbiota.	Young et al., 2020
	Selective effect of pectin on the gut microbiota and SCFAs production.	Bang et al., 2018
Reading model	Impact of prebiotics on gut bacterial proteolysis in a host diet-dependent way.	Wang et al., 2020
	Effects of crystalline polymorphism of resistant starch (RS) type III on the fermentability of RS by the human intestinal microbiota and the production of short-chain fatty acids.	Lesmes et al., 2008
	Effect of probiotics, prebiotics and synbiotics on the elderly faecal microbiota.	Likotrafit et al., 2014
TIM-2	Gut microbiota conversion of polyphenols in predigested mango "Ataulfo" peel.	Sáyago-Ayerdi et al., 2021
	Effect of predigested fructans on gut microbiota.	Sáyago-Ayerdi et al., 2020
SHIME®	Effect of an infant cereal with probiotic on intestinal microbiota.	Salgaco et al., 2021
Baby-SPIME	Effect of AP (apple pomace) on fermentation products	Dufourny et al., 2022
SIMGI	Relationship between apparent viscosity of chia mucilage and human intestinal microbiota.	Tamargo et al. 2018
	Study of the impact of red wine on colonic metabolism.	Cueva et al. 2015
	Study of the interaction of food microplastics and the colon microbiota	Tamargo et al., 2022
PolyFermS	Evaluation of the modulating effect of fermentable dietary fibers (DFs) on two distinct microbiotas of the adult human proximal colon, independently of the host.	Poeker et al. 2018
	Effect of nucleotides and nucleosides on the infant gut microbiota.	Doo et al. 2017
MiniBio	Development of a highly bio-relevant but generic *in vitro* digestion system that simulates the aged intestine.	Levi and Lesmes 2014

TSI	Development of a small volume *in vitro* model with increased throughput focusing on simulating passage through the stomach and small intestine (SI).	Cieplak et al. 2018
Mimicol	Metabolization of the model drug sulfasalazine	Beeck et al., 2021
Arcol	Effect of fasted or fed state on the survival kinetics of the new probiotic Saccharomyces cerevisiae strain CNCM I-3856 and to assess its influence on intestinal microbiota composition and activity	Cordonnier et al., 2015
M-Arcol	For the evaluation of FMT (Faecal Microbiota Transplant) For the evaluation of microplastics interactions with the colon microbiota	Blanque-Diot et al., 2021 Fournier et al., 2023
DGID-CF	Impact of High-Pressure Processed Onion	Fernández-Jalao et al., 2021

1.3.1. Batch fermentation models

Since they are distinguished by a confined anaerobic environment and short-term simulation, batch fermentation models (BFM) are the most straightforward, adaptable, and accessible. The fundamental characteristic of this kind of model is that, due to the exhaustion of the substrate and the buildup of hazardous metabolites, fermentation can only be sustained for brief periods of time. From closed vials inoculated with specific microbial species to controlled reactors inoculated with fecal suspensions, BFM can range in complexity. BFMs are frequently used to investigate how a substance interacts with the intestinal microbiota. A heating plate for temperature control, the administration of acids or alkalis to maintain a stable pH, and the bubbling of N2 on a base chemical media to ensure anoxic conditions and nutrition are used to manage the ecological conditions in more complex batch systems. Batch fermentation studies have explored the potential of a range of edible insects to modulate the human gut microbiota (Young et al., 2020) and have proven the selective effect of pectin on the gut microbiota and SCFA production (Bang et al., 2018). This type of systems has the drawbacks of having limited similarity to *in vivo* conditions, but the advantages of being cost-effective, logistically flexible, and easy to use.

1.3.2. Dynamic fermentation models

Dynamic fermentation models (DFM) provide more accurate simulation of what occurs in vivo, as well as longer-term investigation of the impact of dietary components on the intestinal microbiota and a more complex ecology of the many ecological niches of the GIT. The gut microbiota is continuously fed in DFMs, and ecological parameters are meticulously upheld. Most DFMs were

inspired by the model originally established at the University of Reading (Gibson, Cummings and Macfarlane 1988), consisting of three vessels connected in line to mimic the ecological niches of the three regions of the colon, i.e., proximal, transverse colon and distal (**Figure 1**). Blowing N2 or O2 ensures microaerophilic and anoxic conditions, which are then managed by dissolved oxygen (DO) sensors or mechanical stirrers. The pH can be impacted by CO2, hence N2 is generally recommended. Because of the ample nourishment and somewhat acidic environment, the first vessel is characterized by a rapid development of the microbiota, similar to what occurs *in vivo* in the proximal colon. However, due to neutral pH and a lack of sustenance in the following two vessels, the microbiota's growth is slowed down, just as it is in the distal portions of the colon *in vivo*. Through the entire system, the microbiota, derived from a fecal sample, is kept vital thanks to the maintenance of the temperature and the use of a basal nutrient medium. This dynamic model was used by Wang et al. (2020) to show that prebiotics suppress gut bacterial proteolysis in a host diet-dependent way. In omnivore models, proteins with prebiotic addition increased *Bacteroides spp.* and inhibited *Clostridium cluster IX*, but in vegetarian models, high protein plus prebiotic treatment increased *Clostridium cluster IX* and decreased *Bacteroides spp*. The Reading model was also used by Lesmes et al. (2008) and Likotrafit et al. (2014) to study the effects of crystalline polymorphism of resistant starch (RS) type III on the fermentability of RS by the human intestinal microbiota and to study the effect of probiotics, prebiotics, and symbiotics on the elderly faecal microbiota, respectively.

Due to potential variations in parameter settings, this more complicated system has limited reproducibility but has the virtue of being practical, adaptable, and simple to operate.

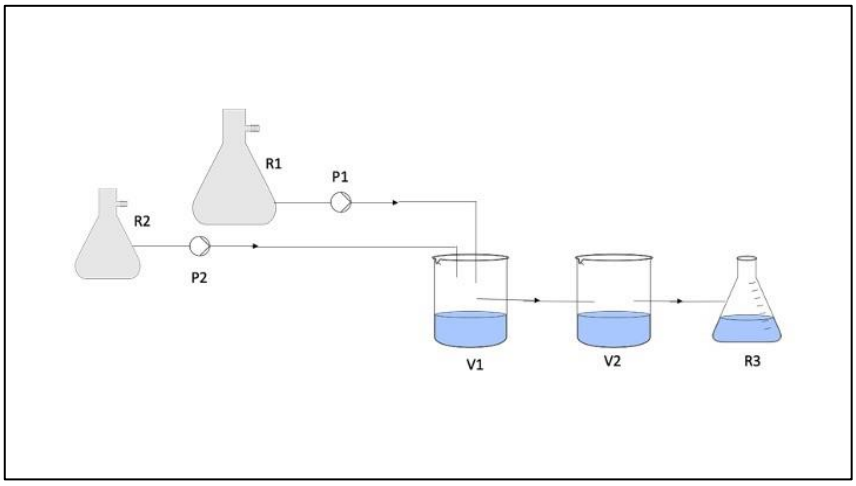

Figure 1. Reading model (adapted from Gibson et al. 1988)

1.3.2.1. The TNO in vitro model of the colon (TIM-2)

The Netherlands Organization for Applied Research, based in The Hague, created the TIM-2, a patented in vitro model of the colon. Along with TIM-1 model, which replicate the ecological settings of the stomach and small intestine, it can be used to simulate the complete human gastro-intestinal tract. The TIM-2 model, which we will mainly discuss here, simulates the proximal colon, which is separated into four functional compartments. Computers control most of the variables that control system settings. The program also regulates the absorption of water and microbial metabolites using a semipermeable membrane and a dialysis system, allowing the hazardous metabolites to be eliminated by avoiding their accumulation in addition to the traditional environmental parameters. The software also enables you to observe the release, bioaccessibility, and interactions of the investigated substrate in each compartment of the model at the conclusion of the experiment. A membrane that simulates a peristaltic movement and a flow of warm water that circulates inside each of the four compartments of this model also allow for the mixing and passage of intestinal fluids. Human fecal suspensions are used to introduce GM into the model (Maathuis et al. 2009; Reimer et al. 2014), and a special medium known as SIEM (simulated ileal efflux media), which contains complex carbohydrates, non-digestible proteins, residual bile, minerals, and vitamins, promotes the growth of the GM (Venema et al., 2000; van Nuenen, Meyer and Venema 2003). The redox potential of the system is kept at roughly -300 mV, similar to that of the human colon, thanks to the development of the microbiota and the bubbling of N2. By adding NaOH, which neutralizes the acids created by the microbiota's metabolism, the pH is maintained at 5.8, which is the typical pH of the proximal colon. The bacteria must first undergo an adaptation period that lasts roughly 16 hours before the 72-hour experiment can begin.

This model was used to study the gut microbiota bioconversion of polyphenols in predigested mango “Ataulfo” peel and to explore changes in gut microbiota with predigested fructans (Sàyago-Ayardi et al., 2021; Sàyago-Ayardi et al., 2020). Despite being extremely reproducible, appropriate for tiny laboratory settings, and achieving a high degree of resemblance with what happens *in vivo*, this model is time- and labor-intensive and expensive, making it inaccessible to most people.

1.3.2.2. The simulator of the human intestinal microbial ecosystem (SHIME®)

The Simulator of the Human Intestinal Microbial Ecosyatem (SHIME) is a system patented under the aegis of ProDigest and the University of Ghent. The SHIME model, which mimics the whole gastrointestinal tract from the stomach to the distal colon, is made up of five reactors arranged in series (Van den Abbeele et al. 2010). Peristaltic pumps are used to link the double-jacketed glass

containers that make up the reactors (Figure 2). A nutrient medium is fed into the first reactor, which represents the stomach, through a filling and withdrawal system, and pancreatic and biliary juices are fed into the second reactor, which represents the small intestine (Venema and Van den Abele 2013). Each of the colon's three parts is divided into compartments with various working volumes—500, 600, and 800 ml—with pH levels that closely match those observed *in vivo* (Van den Abbeele et al. 2010). To get the microorganisms to accurately represent the typical microbiota of the human colon, the system is injected with a fecal suspension from healthy donors, which takes around 14 days to adapt to *in vitro* settings (Van den Abbeele et al. 2010; Venema and Van den Abbeele 2013). This model was used to evaluate the effect of an infant cereal with probiotic on infant intestinal microbiota (Salgaco et al., 2021). This type of system takes time and effort and is not affordable in terms of cost, nor is it suitable for small laboratories, but it is probably the one that achieves the highest level of similarity *in vivo*.

By including a separate segment for the microbiota connected to the mucin layer present on the intestinal epithelium, the SHIME model was transformed into the M-SHIME model (Figure 3) (Van den Abbeele et al. 2011b).

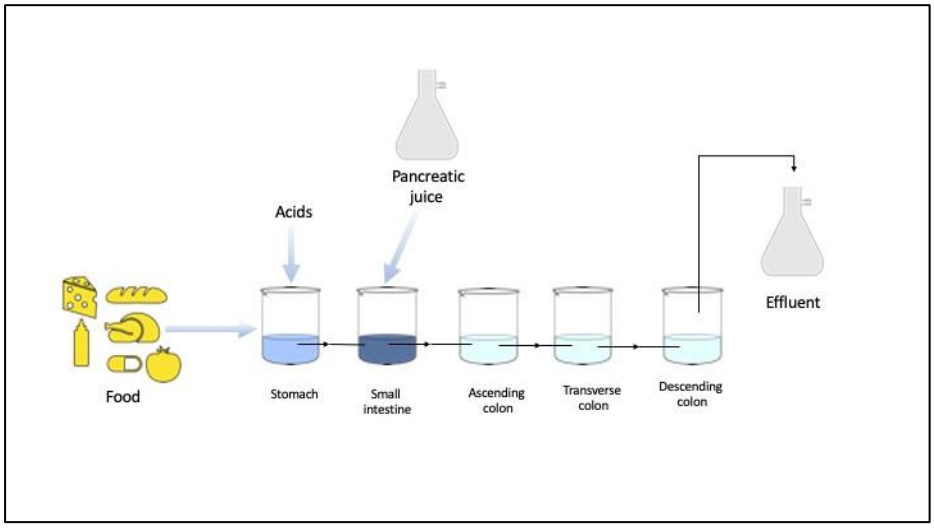

Figure 2. Schematic representation of the SHIME (adapted from https://prodigest.eu/technology/shime/).

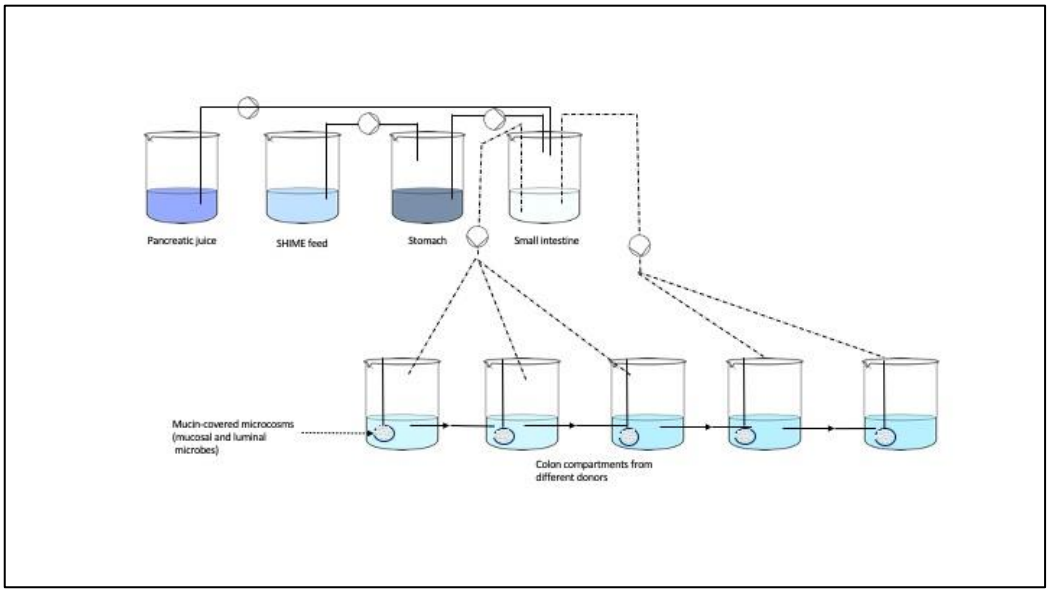

Figure 3. Schematic representation of M-SHIME (adapted from Van den Abbeele et al. 2013)

1.3.2.3. Baby-SPIME

The baby-SPIME model was built using a SHIME® device (ProDigest Bvba, Gent, Belgium), with certain modifications (Dufourny et al., 2019).

The cabinet, in instance, was split into two separate pieces, each housing three bioreactors. The digestion of the stomach and duodenum/jungle was replicated in Bioreactor 1, which was not inoculated. Pig feces were used to inoculate bioreactors 2 and 3, which then mimicked the ileum and proximal colon, respectively. A total retention time of 14 hours is used to determine the three feeding cycles that are scheduled each day. 140 ml of culture media, kept at a temperature of 4 °C, was pumped into bioreactor 1 for one hour and thirty minutes throughout each cycle. Then, pancreatic juice + bile (60 ml), also maintained at 4 °C, was added to the same bioreactor for 1 hour, after which the contents of bioreactors 1, 2 and 3 were simultaneously flowed into bioreactors 2, 3 and one reject, respectively. The flow rates served two purposes: to completely empty bioreactor 1 (200 mL to 0 mL) and to achieve a dwell duration of 4 h and 10 h in bioreactors 2 (constant volume of 100 mL) and 3, respectively. For the ileum bioreactor, the minimum required volume was used to account for the emptying of the small intestine that occurs in vivo, while maintaining a good fermentation process in the bioreactor. The baby-SPIME model for the colonic bioreactor was developed using the same volume as the SHIME model. All bioreactors were flushed with nitrogen (N2) once a day for 10 minutes to keep them in anaerobic condition. The bioreactors were also kept at 39.5°C while being continually stirred (300 rpm). The pH of bioreactors 2 and 3 was continuously monitored by pH adjusters, which used HCl (0.5 M) or NaOH (0.5 M) to maintain pH ranges of [5.80-6.05] in bioreactor 3 (proximal colon) and [6.40-6.60] in bioreactor 2 (ileum) (0.5 M).

1.3.2.4. The simulator gastro-intestinal: SIMGI

A multi-compartmental model called the SIMGI was created in Spain at the Food Science Research Institute (CIAL-CSIC-UAM, Madrid, Spain) to simulate the full human gastro-intestinal system (Barroso et al. 2015). These five reactors are linked together by peristaltic pumps and are operated by a PC using specialized software (Figure 4). The three regions of the large intestine and the stomach are each represented by one of the five components. Two methacrylate modules suspended over a tank and divided by a jacket make up the gastric unit. The shirt stirs the contents while the tank catches the stomach juices. The medium is churned inside the glass reactors that make up the other four units using magnetic stirrers. The biological conditions in each compartment fluctuate in this system, as they do in the other DMFs, and are characterized by defined pH and atmosphere values. An experiment can last up to 6 days, depending on the study that will be conducted (Barroso et al. 2015). SIMGI studies demonstrated the effect of chia seed mucilage (Tamargo et al., 2018) and red wine (Cueva et al., 2015) on the human gut microbiota. SIMGI has the cons of being time-consuming and less realistic and reproducible due to less stringent environmental parameters, but the advantages of being cost-effective, logistically flexible, and easy to use.

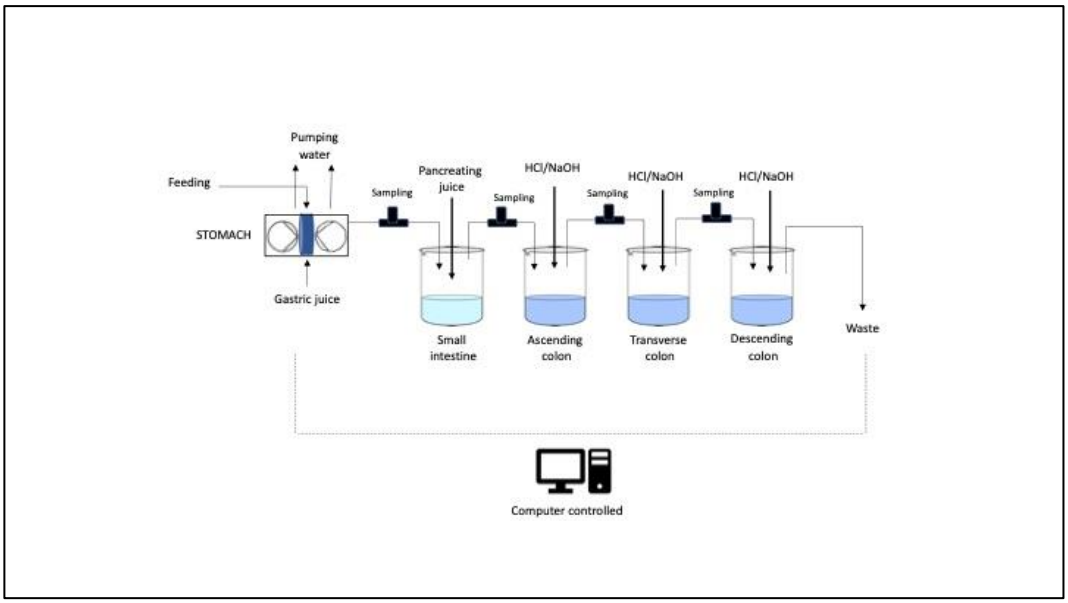

Figure 4. Schematic diagram of the SIMGI (adapted from Barroso et al. 2015)

1.3.2.5. Polyfermentor intestinal model (PolyFermS)

The human gut microbiota is replicated in all of the models discussed thus far using a fecal inoculum. This approach, however flexible and helpful, has been criticized because it ignores the various methods that microbes employ to colonize the intestine of their hosts, such as adherence to binding

sites. Because of their stronger resistance to hunger, ecological displacement, and/or rapid development, some bacteria are able to impose themselves on the simulated microbial community, making it unstable and ruled by them. As a result, the less aggressive microorganisms lose their competitive edge in the struggle for the substrate, which reduces the model's realism. A approach was created that involves capturing the fecal microbiota using the microencapsulation technology in order to get around these restrictions and guarantee a high degree of variety and species abundance over lengthy tests (Le Blay et al. 2010; Zihler et al. 2011; Payne et al. 2012; Dostal et al. 2013). The PolyFermS model (Figure 5), a DFM built at the ETH in Zurich under a joint European mandate, was the first model to use this immobilization procedure (Zhiler-Berner et al. 2013). The model can sustain a stable microbial community over a 38-day stress test and has a separate microbial inoculum in each of the five reactors. Additionally, the artificial microbiota retains the primary bacterial groups during the studies in terms of both diversity and abundance, making it similar to the fecal microbiota of a healthy donor (Zihler-Berner et al. 2013). The model consists of a micro-encapsulated GM inoculum reactor for the upper proximal colon. This reactor's contents are utilized to continually feed the parallel-connected downstream reactors, such as experimental and second-stage reactors, which run in accordance with the proximal colon's ambient conditions. Responses to experimental characteristics are monitored using second stage reactors as a control (Zihler-Berner et al. 2013; Poeker et al. 2018). PolyFermS was used to evaluate the gut microbiota-modulating effects of fermentable dietary fibers (Poeker et al., 2018) and to study the impact on infant gut microbiota of some components of infant formula which imitate the content of breast milk (Doo et al., 2017). Although it takes time and effort, PolyfermS eventually seems to be quite stable, improving parallelism *in vivo.*

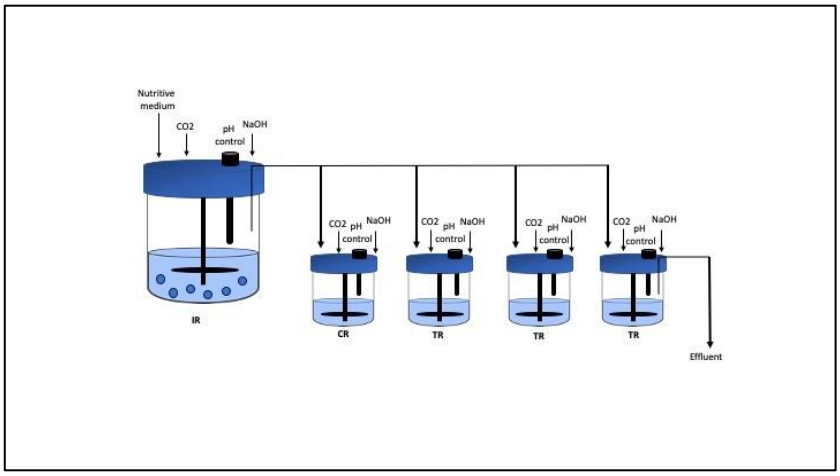

Figure 5. Design of the Polyfermentor Intestinal Model (PolyFermS): effluents of the inoculum reactor (IR) were used to feed a set of second-stage control (CR) and test (TR) reactors (adapted from Zihler Berner et al. 2013)

1.3.2.6. Small scale reactors

Applikon Biotechnology (Delft, Netherlands) recently introduced the patented Mini-Bio *in vitro* model for use in practical research. Mini-Bio is a particularly flexible model since it can be used as both a BFM and a DFM, producing a large amount of data that can be examined and archived using specialized software. To replicate tests that have already been carried out under the identical conditions, the system can directly recreate the settings of the individual experiments from a database (O'Donnell et al. 2018). This system's key novelty is its capacity to remotely manage up to 32 parallel bioreactor fermentations. The bioreactors have a varied working volume, starting from a minimum of 50 ml. The technical characteristics thanks to which the high performance of these systems are possible are: i) mechanical impellers that ensure agitation up to 2000 rpm, allowing the cultivation of high-density cell cultures or viscous substrates; ii) antifoam system with level sensors; iii) gas dispensers; iv) digital pumps with adjustable speed or micro valves (microliters); v) PID adaptive selectable autotuning control that regulates the change of settings during the experiment; vi) liquid-free perfused peltier device for rapid temperature control and regulation; vii) electric condenser to regulate the temperature; viii) optical micro camera to monitor the confluence of cell culture (Lattermann and Bu¨chs 2015). Mini-Bio systems have the disadvantage of being more expensive but the benefits of flexibility, logistical suitability under a lab environment, and good reproducibility because of meticulous parameter tuning and control.

1.3.2.7. The smallest intestine in vitro model (TSI)

Five reactors with a minimum volume of 12 ml each make up the TSI model, a DFM that simulates the passage through the small intestine (Cieplak et al. 2018). Each reactor is housed inside a PVC chamber, and the entire assembly is put into a box whose temperature is controlled by the flow of water that has been heated by a thermostatic coil. With the use of the traditional anaerobic catalyst or by bubbling N2, anaerobic conditions can be created. A plate magnetic stirrer that is separated into five parts, one for each reactor, keeps the medium stirred. A dialysis chamber mimics nutrition absorption. The reactors are insulated with a septic lid, which also serves as an inlet and exit for the dialysis chamber, a pH probe, a needle for introducing pancreatic juices, and sample ports (Cieplak et al. 2018). The essential parameters are computer-controlled; for instance, the pH is continuously monitored, and alkalis and acids are dosed automatically thanks to a specialized Matlab script (The MathWorks, MA). To simulate the electrolyte content and osmotic pressure that occur *in vivo*, distinct artificial gastric and intestinal fluids are employed (Minekus et al. 2014). Cieplak et al. (2018) tested

in the TSI model the behavior of three putative probiotic *Lactobacillus* strains, and by means of culture-dependent microbiology demonstrated the strains' survival passing through the upper GIT. The TSI model has the drawbacks of being less realistic and reproducible due to less stringent environmental settings, but the advantages of being logistically flexible and user-friendly.

1.3.2.8. Mimicking the Dynamic Colonic Microbiota (MimiCol)

MimiCol, a recently created dynamic bioreactor, was created with the intention of reflecting the dynamic circumstances in the proximal large intestine (Beeck et al., 2021). A shorter compendial borosilicate glass vessel with a small cylinder top that was sealed gas-tight with a Teflon head plate made up this innovative bioreactor. The container could hold up to 200 mL. A stepping motor was used to move two perpendicularly oriented PTFE blades at a specific speed and amplitude. To create an anaerobic environment, nitrogen was continually pushed into the reactor headspace. With certain sensors and electrodes, temperature, pH level, and redox potential were continuously measured. A temperature sensor was fitted to regulate the temperature inside the medium, which was kept at 37 °C using a water bath. The reactor was connected to two separate micro-dosing pumps that could provide either 1 M HCl or NaOH solutions. This configuration made pH adjustment automatic. The growth medium for anaerobic bacteria was Schaedler broth pH 7.4. Microbial fermentation activities generate a pH drift toward acidic pH values when they are operating normally. A certain amount of sodium hydroxide solution was automatically supplied if the pH value exceeded the lower specified value of 5.95 and continued to be added until the higher required value of 6.45 was reached. The LabView® software was used to control the entire system. The small intestine's peristalsis frequencies are 15 to 18 minutes for spreading movements and 12 minutes for stationary (mixed) motions (Avvari, 2020). Although frequencies are lower in the colon, compared to the transverse or descending colon, the ascending colon is more similar to the condition of the small intestine (Beeck et al., 2021). To achieve a uniform distribution yet physiological movement inside the reactor, the agitation was set to 6 reciprocations per minute (rpm) with a 90° amplitude. The reactor was filled with a total volume of 140 mL of Schaedler broth and a freshly thawed sample of cryo-preserved standard microbiota, which was washed by centrifugation at 6000 rpm for 6 min and redispersed in 10 mL of Schaedler broth. An injection port was used to introduce the standard microbiota, which had an overall count of roughly 1.5×10^{10} CFU and was primarily composed of obligate anaerobes. The medium was replenished every 2 hours by taking out 135 mL and adding fresh Schaedler broth in its place.

1.3.3.0. ARCOL

ARCOL is a one-stage semi-continuous fermentation system (Applikon, Schiedam, The Netherlands) that incorporates the key elements of the in vivo human colonic environment, such as pH, body temperature, ileal effluent supply, retention time, anaerobiosis maintained solely by resident microbiota activity, and passive uptake of water and fermentation metabolites (Cordonnier et al., 2015). The faecal inoculum is quickly transferred to the bioreactor, flushed with O2-free N2 gas, and made up to 450 mL with culture media after being prepared in a vinyl anaerobic chamber (Coy, Grass Lake, MI, USA) under strictly anaerobic conditions. With a set temperature of 37°C, a controlled constant pH of 6.3, a mean retention duration of 36 hours, and a redox potential (Eh) of -400 mV, the ARCOL model is run under conditions that mimic a healthy human colon. To closely simulate the composition of ileal effluents, the nutritive medium was delivered progressively into the bioreactor and contained various types of carbohydrates, proteins, lipids, minerals, and vitamins (Macfarlane et al., 1998). The dialysis system in the model uses hollow-fibre membranes with a molecular mass cut-off value of 30 kDa, allowing for the maintenance of proper electrolyte and metabolite concentrations as well as operating volume.

Starting from the ARCOL system, the Mucosal Artificial Colon (M-ARCOL) was developed, coupled with a co-colture of intestinal epithelial and mucus-secreting cells. The M-ARCOL model was used by Blanque-Diot et al. (2021) for the evaluation of FMT (Faecal Microbiota Transplant) and by Fournier et al. (2023) for the evaluation of microplastics interactions with the colon microbiota.

1.3.3.1.DGID-CF

The AINIA Technology Centre (Valencia, Spain) created an in vitro dynamic model system (DGID-CF) that can simulate the complete digestion process, including stomach digestion and colon fermentation (Fernández-Jalao et al., 2021). To replicate the physiological conditions that exist during digestion in the stomach, small intestine, ascending colon (AC), transverse colon (TC), and descending colon, the system comprises of five vessels (DC). Peristalstic pumps that work continuously in the three regions of the colon and semi-continuously in the stomach and small intestine link all vessels and shield them from light. The conditions of the entire process, including pH values, residence duration, temperature (37 °C), and volumetric capacity, are computer-controlled. The five vessels are exposed to 150 rpm of agitation. Anaerobiosis is maintained by flushing with gaseous N2 for 15 minutes twice a day. The digestive circumstances in the gastrointestinal system (stomach and small intestine) are comparable to those reported by Minekus et al. (2014). After the small intestine digestion (6 hours) and gastric digestion (2 hours) are finished,

the ascending colon reactor incorporates intestinal digestion three times per day. Colonic tubes are filled and preconditioned with nutritional medium in a volume of 1000 mL in the AC, 1600 mL in the TC, and 1200 mL in the DC before being inoculated with 20 mL of human faecal slurry (20%, w/v), which is made by combining fresh feces with sodium phosphate (pH 7) (Fernández-Jalao et al., 2021). The composition of 1 litre of nutrient medium was: 1 g arabinogalactan, 0.2 g apple pectin, 1 g xylan, 3 g potato starch, 0.4 glucose, 3 g yeast extract, 1 g peptone, 4 g mucin, 0.5 g L-cysteine, 1.5 g NaHCO3, 0.69 g MgSO4-H2O, 0.5 g KH2PO4, 1.5 g MgSO4-H2O, 1.5 g MgSO4-H2O, 1.5 g MgSO4-H2O 5 g KH2PO4, 0.5 g K2HPO4, 0.08 g CaCl2, 0.005 g FeSO4-7H2O, 0.002 g MnSO4-H2O, 0.0006 g ZnSO4-7H2O, 0.0001 g CoSO4-6-6H2O, 0.0001 g MnSO4-H2O. 0001 g CoSO4-6H2O, 10 µL vitamin K1, 1 mL Tween 80 and 4 mL resazurin solution (0.025%, w/v) as anaerobic indicator and distilled water. Before being put into the three colon reactors, the medium is sterilized at 121 °C for 15 minutes, and oxygen is removed by flushing with nitrogen. Each reactor's medium is calibrated and controlled to have a pH of 5.5–6 for AC, 6-6.4 for TC, and 6.4-6.8 for DC, and is kept anaerobic by constant N2 flushing. There are two stages to the experimental process. 12 days are needed for the initial stabilization (basal) phase, during which the gut flora can adjust to the dietary and physicochemical circumstances in the three colon arteries (AC, TC and DC). To feed the system during this time, 200 cc of nutrient medium are fed to the stomach reactor three times per day. The test sample and 200 cc of nutrient medium that simulates a chronic intake are added to the stomach vessel once daily for 14 days during the second phase, which is the intake period. Additionally, the system is only fed 200 ml of nutritional medium twice a day. The volume and transit time of each compartment of the DGID-CF, simulating in vivo residence time, are: 260 mL and 2 h in the stomach, 460 mL and 3 h in the small intestine, 1000 mL and 20 h in the AC, 1600 mL and 32 h in the TC and 1200 mL and 24 h in the DC.

1.3.3.2. Mini Colon Model (MiCoMo)

The Mini Colon Model (MiCoMo) is a low-cost, miniature multi-bioreactor device that replicates the human colon and can adapt culture conditions to suit physiological circumstances or particular experimental requirements (Jin et al., 2022). Anoxia, pH, temperature, and media feeding schedule are just a few of the physiological conditions that can be automatically controlled by MiCoMo and customized by the user. The system doesn't require an anaerobic chamber to function because of its limited working volume. Three single stage reactors with a total capacity of 55 mL and a working volume of 30 mL make up MiCoMo. Each individual reactor has a gas sparging line for N2 flushing to maintain anoxia, as well as an acid/base adjustment and fluidic transfer tube with Luerlock connectors. The N2 sparging, which also serves as MiCoMo's mixing system, homogenizes the

reactor's contents. The anaerobic reactors can be confined in a biosafety cabinet while MiCoMo is in operation, keeping them at 37°C in a water bath to prevent any possible contamination.

1.3.3.3. Copenaghen Colon Model (CoMiniGut)

Five parallel stirred, single-vessel, anaerobic reactor units with pH monitoring and control make up the CoMiniGut prototype (Wiese et al., 2018). A fused quartz glass vial is contained in a 150 ml polymethylmethacrylate (PMMA) compartment in each anaerobic reactor unit. Either Anaerogen compact sachets placed inside the PMMA compartments or integrated gas in- and outputs for flushing the compartments with nitrogen (99.8%) to sustain anaerobiosis are used to create anaerobic conditions (this also facilitates gas or headspace sampling). Anaerobiosis is shown by indicators soaked in resazurin. A PMMA ring and a replaceable vacuum-greased silicon rubber septa with a pH probe inlet make up the lid of the PMMA compartments. Needles can pass through the rubber septa to sample, adjust pH, and, if required, feed substrate. A magnetic stirrer with five stirring locations is the foundation for the parallel alignment of the five reactor vessels in a single unit. A flowing water bath coupled to a heat-exchange plate within the climate box and an external temperature probe placed inside the box for feedback control keep the temperature inside the climate box at 37 °C. An uniform temperature distribution is ensured through a ventilation system, and temperature logging (using a Temp 101A MadgeTech Temperature data logger) is done throughout trials. Using a 6-channel pH meter and data recorder, the pH is tracked (Consort multi-parameter analyser C3040). A multichannel syringe pump that is loaded with syringes holding 1 M NaOH is controlled by a multichannel syringe pump that is connected to a laptop running custom Matlab programs for pH control. The injection needle and tubing (VWR) from the syringes are attached to the fermentation compartments.

1.3.3.4. MiGut

To produce a scalable colon simulator with a better experimental throughput than other models, the Mini Gut (MiGut) platform builds on current in vitro technology. MiGut preserves clinical relevance while preserving all of the capability of other fully instrumented three-stage models, in contrast to other scaled systems (like MBRA).

It is feasible using this platform that hundreds of in vitro models can be performed concurrently, allowing for considerably more extensive research of complicated microbiome-xenobiotic interactions, together with high-throughput molecular approaches for sample analysis. MiGut is a special platform that enables the simultaneous execution of several colonic models with little resource consumption and added complexity to help our comprehension of the cause-and-effect connections

that control the gut microbiota. By using this model system, it is now possible to provide more clinically useful data.

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2. AIM AND STRUCTURE OF THE PhD THESIS

After the above general introduction on the main aspects related to the treated topics, in this section the aim and the manuscript structure of this work are reported. Afterwards, the different case studies presented as scientific papers (someone already published, others just submitted or planned for publication in scientific journals). While specific material and methods, results and discussion together with conclusions are treated within each case study, a chapter for the whole conclusions and one with final remarks and future perspectives ended this manuscript.

Over the past 30 years, unhealthy diets and lifestyles have increased the incidence of non-communicable diseases and led the overweight and obese population in most European countries approach epidemic proportions (Belc et al., 2019). To remedy this, the food industry has turned to reformulating certain foods, leading to the spread of fortified foods, substitute ingredients, supplements, etc. Another important cause in the changes in eating habits is the increase in food intolerances to specific food components, such as gluten and lactose, as well as the spread of the perception that a diet without these components is healthier, which causes these foods to be consumed more and more frequently even by healthy individuals other than those for whom these foods were developed.

The ultimate goal of this work is therefore to provide knowledge on the effects on the intestinal microflora of new foods that are becoming increasingly popular on the world market, not only for specific consumer groups with food intolerances or allergies, but also to consumers attracted by the idea that a diet without or rich in specific components constitutes a healthier diet.

In order to achieve these objectives, the actions taken were: i) to develop a colonic fermentation protocol using a biofermenter; ii) to compare innovative food (or ingredient) prototypes with similar standards or commercial products; ii) to test the effect of different formulations or processes on prebiotic and probiotic characteristics.

The experimental activities consisted of eight case studies. They are representative examples of the most common substitutions/additions/fortifications in the area of dairy products, meat products and vegetable products, as well as specific ingredients containing prebiotics categories commonly used.

Table 5 reported the list of case studies for each topic and the related Chapter.

TOPICS	**CASE STUDY**	
REFORMULATED FOODS	**CASE STUDY 1**: Colonic *in vitro* model assessment of the prebiotic potential of bread fortified with polyphenols rich olive fiber	**Chapter 4**
	CASE STUDY 2: Bread fortified by spirulina	**Chapter 5**
	CASE STUDY 3: Alternative formualtions to mitigate the nasty impact of commercial salami on colon microbiota	**Chapter 6**
FOOD SUPPLEMENTS	**CASE STUDY 4:** Multiunit *In Vitro* Colon Model for the Evaluation of Prebiotic Potential of a Fiber Plus D-Limonene Food Supplement	**Chapter 7**
	CASE STUDY 5: Beneficial metabolic transformations and prebiotic potential of hemp bran and its alcalase hydrolysate, after colonic fermentation in a gut model	**Chapter 8**

LACTOSE-FREE DAIRY PRODUCTS	**CASE STUDY 6:** Colonic *in vitro* assessment of the effect of lactose-free milk on gut microbiota of healthy and lactose intolerant donors	**Chapter 9**
	CASE STUDY 7: *In vitro* study of the effect of fermented whey on colonic microbiota of healthy and lactose intolerant donors	**Chapter 10**
ANTIBIOTICS	**CASE STUDY 8:** Maternal amoxicillin treatment perturbates piglets' colon microbiota	**Chapter 12**

To the first topic (Reformulated foods) belong the first three case studies. The **CASE STUDY 1** aimed to explore the prebiotic potential of bread enriched with Polyphenol Rich Fibre (PRF), with a view to sustainability by exploiting the by-products of industrial processing of natural raw materials. The **CASE STUDY 2** aimed to explore the impact on healthy subjects of gluten-free (GF) bread fortified with *Arhtrospira platensis* powder to enrich the protein content. Nowadays, the target of GF foods goes beyond coeliacs due to the common conception of these products as healthier or suitable for weight loss. Finally, the **CASE STUDY 3** aimed to explore the effect on the colonic microbiota of alternative salami formulations, in which nitrites were replaced by ascorbic acid and/or a mixture of plant antioxidants. Alternatives to nitrites, which in the host can lead to the formation of toxic compounds such as nystrosamines, are increasingly in demand by the food industry to reduce the occurrence of diseases of the gastrointestinal tract.

To the second topic (Food supplements) belong the fourth and the fifth case study. In particular, the **CASE STUDY 4** investigated the prebiotic potential of a new supplement based on fibre and D-Limonene, one of the components of essential oils known not only for its antimicrobial and bacteriostatic activities, but also for its ability to modulate the colonic microbiota. Like the first, the **CASE STUDY 5** is set in the context of food industry sustainability and by-product valorisation, with the aim of assessing the prebiotic potential of hemp seed bran and HB protein isolate treated with alcalase.

To the third topic (Lactose-free dairy products) belong the sixth and the seventh case study. Given the high incidence of lactose intolerance, **CASE STUDY 6** aimed to explore the impact of lactose-free milk on the gut microbiota of both lactose-intolerant and healthy subjects. In fact, all members

of a family often switch to lactose-free products when only one member is intolerant, so it is also interesting to study the effect these products have on consumer groups other than those for whom they were developed. In the same context is **CASE STUDY 7**, which aimed to explore the effect on the colonic microbiota of healthy and lactose-intolerant subjects of delactosed whey. As one of the main by-products of the dairy production chain, the re-use of whey is a challenge for the food industry with a view to sustainability.

Finally, for the last topic (Antibiotics), **CASE STUDY 8** aimed to explore how maternal amoxicillin treatment perturbates piglets' colon microbiota by using MICODE modified inoculating piglet faeces. In the last decades, the swine has been acknowledged as one of the most important preclinical species for a wide variety of physiological patterns. Indeed, the swine species show close similarities with humans, and the employment of pigs in research trials seems to be more widely accepted by society in terms of ethical values.

3. MICODE (Multi-Unit Colon Model) a new *in vitro* colon model

3.1. Setting-up of MICODE

The *in vitro* gut model called "Multi-Unit Colon Model (MICODE)" was obtained through the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by Lucullus PIMS software (Applikon Biotechnology BV, NL). Before each experiment, bioreactors were autoclaved at 121 °C and −1 bar for 15 min and, once cooled, aseptically filled with 90 mL of anaerobic pre-sterilized basal nutrient medium according to previous publications (Connolly et al., 2012). Basal medium (BM) contained (per L): 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K2HPO4, 0.04 g KH2PO4, 0.01 g MgSO4·7H2O, 0.01 g CaCl2·6H2O, 2 g NaHCO3, 2 mL Tween 80, 0.05 g Hemin dissolved in 1 mL of 4 M-NaOH, 10 mL vitamin K, 0.5 g L-cysteine HCl, and 0.5 g bile salts (sodium glycocholate and sodium taurocholate). Before autoclaving, the medium was brought to pH 7.0, and 2 mL of a 0.025% (w/v) resazurin solution were added once the media had cooled. Fermentation vessels were filled aseptically with 90 mL of BM and the bioreactor headplates were mounted on previously sterilized and calibrated sensors, i.e., pH and Dissolved Oxygen (DO2) sensors. Anaerobic condition (0.0 - 0.1% w/v of DO2) in each bioreactor was obtained after about 30 min flushing with filtered O2-free N2 through the mounted-in sparger of Minibio reactors (Applikon Biotechnology BV, NL), and was constantly maintained over the experiments. Temperature was set at body temperature (37°C for human or 39°C for piglets) and stirring at 100 rpm, while pH was adjusted to the desired value (depending on the human or animal colon region mimicked) and kept throughout the experiment with the automatic addition of filtered NaOH or HCI (0.5 M). Once the exact environmental settings were reached, each of the four vessels were aseptically injected with 10 mL of fecal slurry (10% w/v of feces) to a final concentration of 1% (w/v), and then with 1 g of the appropriate substrate/treatment to test for a final concentration of 1% (w/v) (Koutsos et al., 2017). The fourth vessel was set as blank control (BC, basal medium and 1% fecal slurry only). Fresh fecal samples were collected in an anaerobic jar, maintained at 4 °C and processed within 1 h. Fecal slurry was prepared by homogenizing the feces in pre-reduced phosphate-buffered saline (PBS) (Wang et al., 2020). Batch cultures were run under these controlled conditions in three bioreactors (AppliSense Sensors, Applikon Biotechnology BV, NL) reported in **Figure 6** and **Figure 7** for a period of 24 h, during which samples were collected at 3 timepoints (BL = Baseline, T1 = 18 h and EP endpoint = 24 h). The baseline (BL) was defined on the first pH changes (Venema, 2015) detected by sensors (1 read/10 s) via the internal pH Sensors of MICODE. To guarantee a close control, monitoring and

recording of fermentation parameters, the software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) was used. Sampling was performed with a dedicated double-syringe-filtered system connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. This also allowed the stability of all settings to be maintained during the experiment. Fermentations were conducted in duplicate independent experiments.

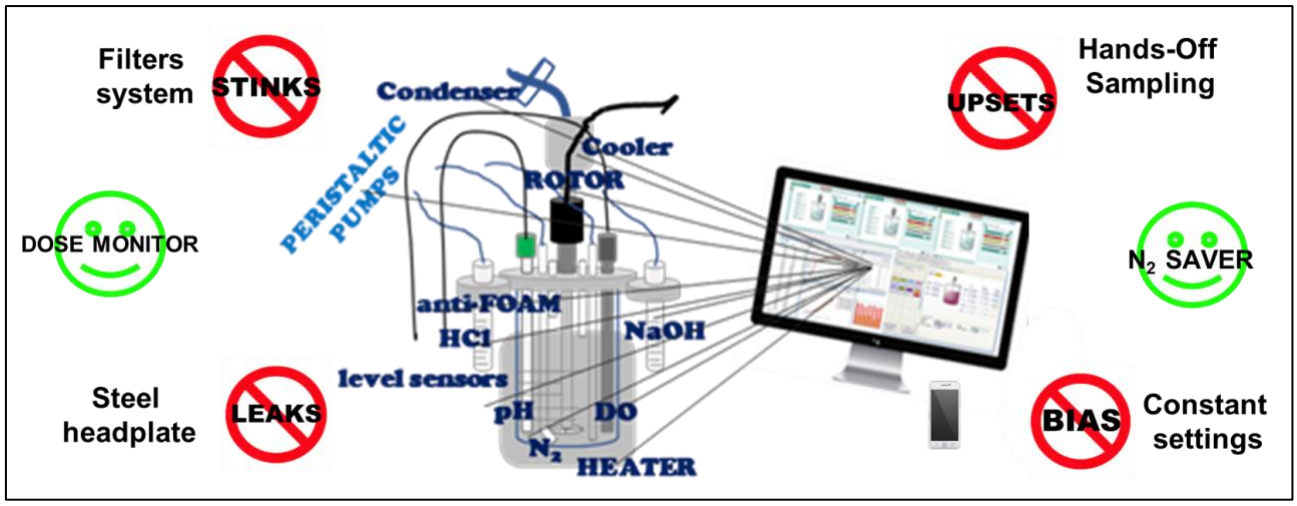

Figure 6. Schematic representation of a MiniBio reactor.

Figure 7. The MICODE model.

3.2. Conceptual and analytical outputs to study the prebiotic potential by MICODE

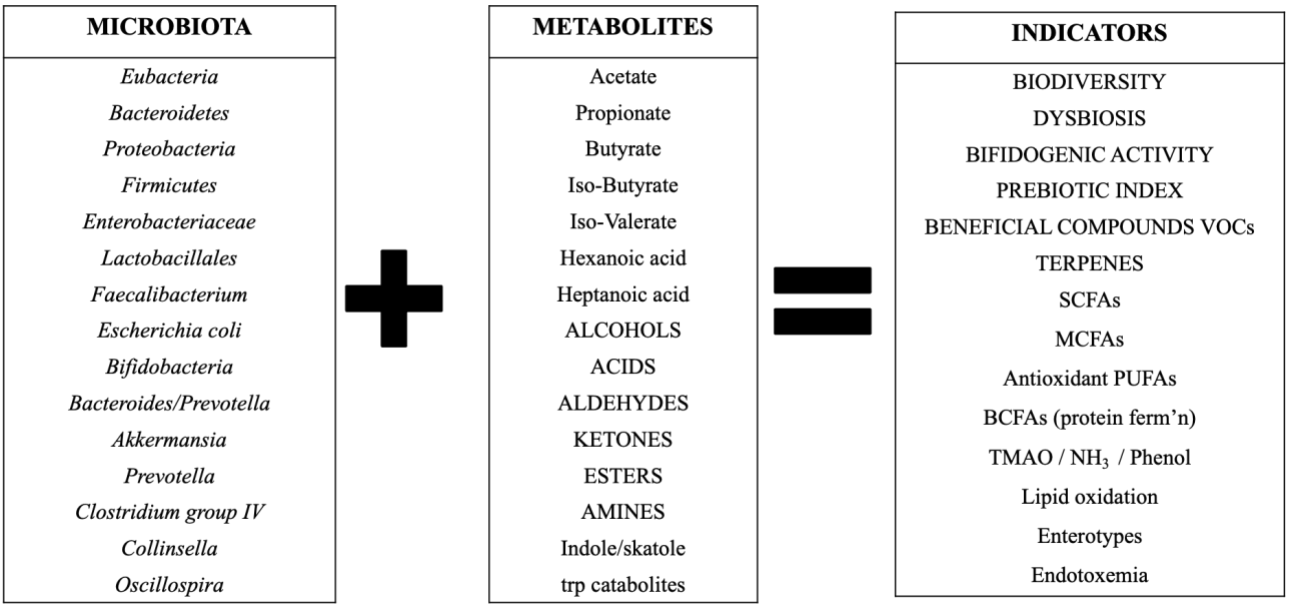

Figure 8. Analytical outputs.

According to scientific literature reported in the "General Introduction" section, for a better data interpretation some analytical outputs were selected as generally accepted as microbial or metabolic signals (**Figure 8**) of gut microbiota perturbations coming from food. Indeed, their changes may be considered closely correlated to the prebiotic potential. In our case, variations in the composition of the microbiota, both at the level of phylum (*Bacteroidetes*, *Proteobacteria*, *Firmicutes*), order (e.g. *Lactobacillales*) or family (e.g. *Enterobacteriaceae*), but also at the level of genus (e.g. *Akkermansia*, *Prevotella*, etc.) or species (e.g, *Escherichia coli*), together with the production of certain microbial metabolites (e.g. free fatty acids, Indole/Skatole, etc.) were selected to determine indicators suitable to predict the impact of a food or a food component on the human colonic microbiota. These indicators include, for example, the prebiotic index, or the production of SCFA or BCFA, beneficial and potentially toxic compounds for the host, respectively. In particular, by the prebiotic index, described in detail in case study 4 (Chapter 7), it is possible to determine the prebiotic potential of a substrate based on its ability to support the growth of beneficial microorganisms and hinder opportunistic ones. Other indicators are, for example, biodiversity and dysbiosis, or bifidogenic activity, i.e. the ability of a substrate to support the growth of the beneficial family of *Bifidobacteriaceae.*

3.3. References

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4. CASE STUDY 1: Colonic *in vitro* model assessment of the prebiotic potential of bread fortified with polyphenols rich olive fiber

(Published in Nutrients. DOI: 10.3390/nu13030787)

4.1. Introduction

Currently, the exploitation of byproducts from industrial processing of natural feed- stocks is a fundamental requisite for sustainability and to contrast pollution generated by synthetic the production of compounds. Polyphenol-Rich Fiber (PRF) is a defatted olive pomace byproduct obtained from olive oil transformation, which was previously exploited under the aegis of H2020 EcoProlive to produce new functional ingredients for bakery foods, thereby increasing their nutritional value (Boubaker et al., 2016). The effects of PRF on the human colon microbiota and its alleged prebiotic potential merit consideration given that it is used as an inventive and affordable technique to create healthier and value-added foods. In fact, it is evident from the most recent definition of prebiotics (Gibson et al., 2017) that molecules other than fructo- or galacto-oligosaccharides could attempt to make a prebiotic claim. Prebiotics have essential characteristics (Sanders et al., 2019) that are expressed toward improving host health, such as altering the microbiota to foster beneficial bacteria while restraining pathogens, as well as producing microbial compounds that are beneficial to the host, such as major SCFAs (Leblanc et al., 2017; Sun et al., 2018) or minor MCFAs (Rial et al., 2016). Prebiotics are by definition degraded by colon microbes and contribute to the modulation of the entire microflora. They do this, for instance, by encouraging the growth of commensals other than probiotics, such as *Bacteroides spp*. (Oba et al., 2020), which are connected to microbial eubiosis and, consequently, to host health (Manor et al., 2020). Similar to this, a prebiotic is meant to modify and limit certain bacterial groups indicated in tryptophan degradation from proteolytic fermentation, whose signature molecules have a deleterious effect on the host, such as BCFAs (Wang et al., 2020) and some indoles (Roager et al., 2018). Because the impact of a prebiotic

on colon microbiota is greater than anticipated, it is essential to have a technique that allows for the investigation of the intricate microbial ecology at play. Prebiotics' effects on the human gut microbiota can be understood using in vitro gut models, which concentrate on the changes in the major microbial groups and specific species and their metabolites while measuring the community's diversity, richness, composition, and abundance over time (Nissen et al., 2020a). In this work, for the first time, we are presenting Multi-Unit *In vitro* Colon Model (MICODE) a versatile *in vitro* colon model, that we are introducing in the version simulating the proximal colon, which is able to resemble the microbe-driven colon fermentations, as happens *in vivo*. We investigated the prebiotic potential of PRF-enriched breads using an interomic approach, which combines microbial genomes and metabolomics, using MICODE with fecal contributions from three healthy donors. Standard breads were enriched with 4% (w/w) PRF and subjected to in vitro gastro-duodenal digestion. Digested fractions were used to reveal and support the prebiotic potential of PRF-enriched bread through the study of ecological indicators, such as: (i) microbial biodiversity, (ii) microbial eubiosis, (iii) prebiotic activity, (iv) the production of desirable compounds, such as SCFAs and MCFA.

4.2. Materials and methods

4.2.1. Fecal donors

Fecal donations were obtained from three healthy donors, two females and one male aged between 30 and 45 y (Wang et al., 2020; Gibson et al., 2017; Connolly et al., 2012; Koutsos et al., 2017). Donors did not undergo antibiotic treatment for at least 3 months prior to stool collection, did not intentionally consume pre- or probiotic supplements before the experiment, and had no history of bowel disorders. Additionally, the donors were normal weight, not smokers, not chronically consuming any drug, and not alcoholic drink consumers. Fecal samples were donated two times (between seven days) for the two biological replicas (Wang et al., 2020; Gibson et al., 2017; Connolly et al., 2012; Koutsos et al., 2017). The three healthy donors were told of the study's aims and procedures and gave their verbal consent for their fecal matter to be used for the experiments, in agreement with the ethics procedures required at the University of Bologna.

4.2.2. Materials

Except where otherwise noted, all chemicals, solvents, and enzymes used in in vitro digestion and batch culture fermentation were of the highest analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Carlo Erba Reagents (CEDEX, Val de Reuil, FR). Reagents for molecular biology (PCR and qPCR), as well as kits for DNA extraction and genetic standard purifications, were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

4.2.3. Experimental breads and controls

Experimental bread prototypes were previously prepared and characterized (Di Nunzio et al., 2018; Minekus et al., 2014). Briefly, 4% (w/w) of PRF was added to baker's yeast-leavened breads, and the PRF-added breads (Eco 4%) were compared to their corresponding controls, i.e., the same bread without PRF (Eco 0%).

4.2.4. In vitro gastric and duodenal digestion

The experimental breads went through *in vitro* digestion. According to the INFOGEST defined methodology (Minekus et al., 2014), the digestion process was carried out on 5 g of bread for 245 min (2 min for oral, 120 min for gastric, and 120 min for intestinal digestion). The addition of simulated saliva (containing 75 U/mL α-amylase), simulated gastric juice (containing 2000 U/mL pepsin) at an acid pH, and simulated pancreatic juice (containing 10 mM bile and 100 U/mL pancreatin) at a neutral pH resulted in a series of successive enzymatic treatments during *in vitro* digestion. The resultant solutions were digested and then frozen at -80 °C pending additional *in vitro* colonic fermentation.

4.2.5. Fecal batch-culture fermentation and samples collection

Colonic fermentations were performed using experimental breads and the prebiotic positive control fructo-oligosaccharides (FOS) from chicory (Sigma-Aldrich, St. Louis, MO, USA). Multi-Unit Colon Model (MICODE). Temperature was set at 37 °C and stirring at 100 rpm, while pH was adjusted to 5.75 and kept throughout the experiment with the automatic addition of filtered NaOH or HCI (0.5 M), to mimic the conditions located in the proximal region of the human large intestine. Once the exact environmental settings were reached, each of the four vessels were aseptically injected with 10 mL of fecal slurry (10% w/v of human feces) to a final concentration of 1% (w/v), and then with 1 g of the appropriate substrate/treatment (FOS, digested Eco4% and Eco0% breads) for a final concentration of 1% (w/v) (Koutsos et al., 2017). The fourth vessel was set to be free of substrate. Four timepoints of samples were taken while batch cultures were running for 24 hours under these controlled circumstances (0, 5, 10, and 24 h). Sampling was performed with a dedicated double-syringe-filtered system connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a close control, monitoring and recording of fermentation parameters, the software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) was used. This also allowed the stability of all settings to be maintained during the experiment. The same

three healthy donors' feces were used in two separate, independent fermentation studies, each employing a fresh pool. Figure S1 reports parameter trends from the experiment.

4.2.6. Pipeline of analytical activities

Samples of the different timepoints were used for qPCR and SPME GC/MS or at 24 h for the 16S-rDNA MiSeq analyses. Specifically, microbial DNA extraction was conducted just after sampling. DNA samples and GC/MS samples were then stored at −80 ◦C. Technical replicas of analyses were conducted in duplicate for SPME GC/MS and 16S-rDNA MiSeq, and in triplicate for qPCR. Statistical analyses were also reported below, in detail.

4.2.7. DNA extraction, amplification, and sequencing

DNA was extracted from each sample at the baseline and the endpoint using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK). The bacterial diversity was obtained by the library preparation and sequencing of the 16S rDNA gene. The following two amplification steps were performed: an initial PCR amplification using 16S locus-specific PCR primers (16S-341F 5′- CCTACGGGNGGCWGCAG-3′ and 16S-805R 5′-GACTACHVGGGTATCTAATCC-3′) and a subsequent amplification integrating relevant flow-cell-binding domains (5′-TCGTCG GCAGCGTCAGATGTGTATAAGAGACAG-3′ for the forward primer and 5′-GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAG-3′ for the reverse overhang), and unique indices selected among those available Nextera XT Index Kits were combined according to manufacturer’s instructions (Illumina Inc, San Diego, CA, USA). Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, USA). In addition, libraries were quality-tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent technologies, Santa Clara, CA, USA). Libraries were sequenced in a MiSeq (Illumina Inc, USA) in the paired end with 300-bp read length (Marino et al., 2019). Sequencing was conducted by IGA Technology Service S.r.l. (Udine, Italy).

4.2.8. Sequence data analysis

Reads were de-multiplexed based on Illumina indexing system. QIIME 1.5.0 was used to analyze the sequences (Caporaso et al., 2010). Operational Taxonomic Units (OTUs) defined by a 97% of similarity were selected using the Uclust v1.2.22 q method (Edgar et al., 2010) after filtering based on read quality and length (minimum quality = 25 and minimum length = 200). The representative

sequences were then submitted to the RDP classifier (Wang et al., 2007) to obtain the taxonomy assignment and the relative abundance of each OTU using the Greengenes 16S rRNA gene database (McDonald et al., 2011). QIIME 1.5.0 was used to conduct assessments of the alpha- and beta-diversity (Caporaso et al., 2010).

4.2.9. Enumeration of bacterial groups

Changes in the *Eubacteria* kingdom, *Lactobacillales* order, *Bifidobacteriaceae*, *Enterobacteriaceae*, and *Clostridiaceae* families, as well as *Escherichia coli* species were assessed by qPCR targeting a small fragment of mono copies or multi copies genes by degenerated or specific MALDI grade primers pairs and high-fidelity DNA polymerase (Invitrogen Platinum SuperFi II DNA Polymerase, Thermo Fisher Scientific, USA) (Table S1). qPCR analyses were performed on a RotorGene 6000 (Qiagen, Hilden, Germany) with the SYBR Green I chemistry. Genetic standards were prepared from relative PCR amplicons of the target bacterial species, using a GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, USA) as described previously (Gibson et al., 2017; Nissen et al., 2019; Tanner et al., 2014). For each of the targets, qPCR reactions were set as follows: a holding stage at 98°C for 6 min, and a cycling stage of 95 °C for 20 s and 60°C for 60 s, repeated 45 times, followed by melting curves analysis. Quantifications were made with five-points standards of the given amplicon separately. Reactions were prepared with 1 ng of DNA, 2x Power up SYBR Green (Thermo Fisher Scientific, USA) and 250 nM of each MALDI grade primers (Eurofins Genomics, Ebersberg, Germany).

4.2.10. Volatilome analysis

Volatile organic compound (VOCs) evaluation was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The Solid Phase Micro-Extraction SPME/GC-MS protocol and the identification of volatile compounds were done according to previous reports, with minor modifications (Nissen et al., 2020a; Saa et al., 2014). Briefly, 3 mL of vessel content or fecal slurry were placed into 10-mL glass vials and added to 10 μL of 4-methyl-2-pentanol (final concentration, 4 mg/L), as the internal standard. Samples were then equilibrated for 10 min at 45 ◦C. SPME fiber, coated with carboxen-polydimethylsiloxane (85 μm), was exposed to each sample for 40 min. Preconditioning, absorption, and desorption phases of SPME–GC analysis, and all data-processing procedures were carried out according to previous publications (Nissen et al., 2020a; Saa et al., 2014).

Briefly, before each head space sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 ◦C in a blank sample. The samples were then equilibrated for 10 min at 40 ◦C. The SPME fiber was exposed to each sample for 40 min, and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 ◦C for 1 min, then programmed at 1.5 ◦C/min to 65 ◦C, and finally at 3.5 ◦C/min to 220 ◦C, which was maintained for 25 min. Temperatures for the injector, interface, and ion source were 250, 250, and 230 C, respectively. Helium was employed as a carrier gas at a rate of 3 mL/min for the split-less injections. By scanning mass spectra in the accessible databases (NIST 11 MSMS library and NIST MS Search software 2.0), compounds were identified (NIST, Gaithersburg, MD, USA). Every VOC was fairly measured in percentages. Besides, in samples prior to in vitro colonic fermentation (baseline) (Table S2) the main microbial metabolites related to prebiotic activity were also absolutely quantified in mg/Kg. Samples at the endpoint (24 h) were compared to the baseline for these latter compounds, and values were expressed as shifts. All findings were presented as the normalized means of duplicate measurements from two separate studies.

4.2.11. Statistical analysis

The QIIME pipeline version 1.5.0 (Caporaso et al., 2010) was utilized for the analysis of the sequencing data. Within-community diversity (alpha diversity) was calculated using observed OTUs, Chao1 Shannon, Simpson, and Good's coverage indexes with 10 sampling repetitions at each sampling depth. To compare the latest sequence/sample values of various treatments within an index, the Student's t-test was used. Analysis of similarity (ANOSIM) and the ADONIS test were used to determine statistical differences between samples (beta diversity) following the QIIME compare categories.py script and using weighted and unweighted phylogenetic UniFrac distance matrices. The QIIME beta diversity plots method was used to create Principal Coordinate Analysis (PCoA) plots (Marino et al., 2019). For the rest of the data analyses, Statistica version 8.0 (Tibco Inc., Palo Alto, CA, USA) was used. For the microbiota, the qPCR analysis, and the volatilome one-way ANOVA was used to determine differences between fermentation treatments (blank control, FOS, Eco0%, and Eco4%) at similar timepoints (0, 5, 10 or 24 h), followed by the Tukey's Honestly Significant Difference (HSD) post hoc test. Principal Component Analysis (PCA) was used to evidence discrimination between communities among treatments and applied to a normalized dataset of significant bacterial species (Bonferroni test $p < 0.05$), while PCA and multivariate ANOVA (MANOVA) were applied to super-normalized datasets of chemical classes of the volatilome. Then, two independently normalized datasets of the relative quantifications of the key metabolites related to prebiotic activity and the microbiota at the species level were coupled, and this resulted in a

Spearman rank correlation dataset that was expressed as a two-way joining heatmap with Pearson dendrograms. When the results were shown as shift, we took them into account in relation to a baseline of values that was derived from the analysis of the fecal slurry diluted in PBS and in the BM with the addition of the test sample (FOS, Eco0%, and Eco4%).

4.3. Results and Discussion

4.3.1. Quality Controls for the Validation of MICODE

To validate the MICODE *in vitro* model in the version of fecal batch of the human proximal colon, we chose to monitor and check some parameters as quality controls, other than the trends of the experimental conditions that were plotted over the experiments by Lucullus 3.1 (Applikon Biotechnology BV, The Netherlands) (Figure S1). Quality controls were both related to metabolites and microbes at the end of fermentations and in comparison to the baseline. Specifically: (i) The *Firmicutes*/*Bacteroidetes* ratio (F/B), which is related to health and disease (Koliada et al., 2017), was maintained at a low level, confirming the capacity to simulate a healthy in vivo condition for 24 h. (ii) The presence of *Archea* (e.g., *Methanobrevibacter smithii*) was retained from stools throughout the experiment in each vessel and repetition, indicating that the environmental conditions were prolonged all over. In fact, *Archea* are renowned for their sensitivity to environmental stressors (Samuel et al., 2007). (iii) The alpha diversity indices of the microbiota were kept similar. For example, the Good's rarity index was unchanged ($p > 0.05$), indicating the ability of MICODE to support the growth of rare and fastidious species, while the Observed OTUs richness index scored more than 400 OTUs at the endpoints. (iv) The paradigm of prebiotics was confirmed; in fact, a massive probiotic and SCFA increase and a minimal depletion of enteropathogens were recorded when FOS was applied on MICODE. (v) Each GC/MS analysis had quantified some stool-related compounds (Urea, 1-Propanol, and Butylated hydroxy toluene), that ranged across the complete chromatogram and were adsorbed at the same retention times. Quality controls on the biodiversity of the microbiota undergoing *in vitro* colonic fermentation were firstly introduced by Takagi and colleagues to confirm that the mixture of microorganisms growing in the in vitro system truly represented the taxonomic diversity of the human microbiota (Takagi et al., 2016).

4.3.2. Changes in Fecal Bacterial Alpha and Beta Diversities

Alpha diversity indicators (Figure S2), such as the number of detected OTUs for richness, the Chao1 estimator for abundance, the Shannon entropy for evenness, the Simpson index for dominance, and the Good's measure for rarity, were used to quantify the diversity of gut microbiota within a community. Along alpha diversity, beta diversity with Bray–Curtis analysis and ANOSIM was

evaluated to consider differences among samples (Figure S3). With the exception of Simpson's and Good's, there were differences between the substrates at the endpoint in relation to the baseline for each alpha diversity index. All indicators dropped in relation to the baseline, despite the Eco0% and Eco4% being comparable to one another ($p < 0.05$). The substrate that decreased all of the indices the greatest was FOS. For example, the decrease in richness and evenness was explained by the trend of dominance that indicated that some OTUs were overwhelming the others, reducing both richness and the uniform distribution of bacteria. Otherwise, the Simpson index was reduced but not significantly ($p > 0.05$). This resulted from FOS's capacity to favor a small number of species, such as probiotics, and make them dominate over the microbiota, hence lowering overall alpha diversity. While FOS lowered the observed OTU index from the baseline by 2.2-fold ($p < 0.05$), Eco4% reduced it by just 1.6-fold ($p < 0.05$), showing a mitigated impact on diversity reduction. In addition, the Shannon indices of FOS and Eco4% were 1.8 and 1.4 times less than the baseline, respectively, when the evenness of the microbiota was considered ($p < 0.05$). The fact that ECO4% showed less ability than FOS to diminish these indices suggested that it had a larger range of bioactivity on more bacterial targets. Even for rare bacterial species, MICODE maintained a steady ecological environment, as evidenced by the Good's diversity index remaining constant from the baseline throughout the fermentations. Principal coordinate analysis (PCoA) based on an unweighted (qualitative) phylogenetic UniFrac distance matrix demonstrated ANOSIM clustering on a time basis when the bacterial diversity between samples (beta diversity) was examined, clustering the baseline cases close to the donors and separately from the endpoint cases. Moreover, beta diversity PCoA included the nearby replicas of cases, indicating scarce experimental variations and significant ANOSIM ($p < 0.01$). The limitation of single-batch in vitro models may be highlighted by a decrease in alpha diversity indices reported in a related study using a related *in vitro* model (Koutsos et al., 2017).

4.3.3. Fecal Bacterial Relative Abundance at the Phylum and Species Level

The total sequence reads used in this study were classified into ten phyla, two other than the previous, and one was unassigned (Table 1). In any tested case, the core microbiota was represented by five main phyla, i.e., *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*, in a descending proportional order. These phyla, along with the less represented *Euryarcheota*, were subject to significant changes over colonic fermentation in comparison to the baseline ($p < 0.05$). It is critical to emphasize the trend of the ratio *Firmicutes/Bacteroidetes (F/B)* over time in order to demonstrate the prebiotic potential of Eco4%. In fact, this ratio denotes a microbiota eubiosis when it is between 1.5 and 2, and a dysbiosis when it is greater than 2, which causes intestinal syndromes (Koliada et al., 2017; Zhou et al., 2017). Fecal samples from this investigation had an *F/B* ratio of

about 1.6 at baseline, indicating that the donors were in good health. This ratio remained constant following fermentation with either FOS (1.7) or Eco4% (1.3), but it was 2.3 times greater when colonic fermentation was carried out with Eco0% ($p < 0.05$). These results supported the beneficial effects of polyphenol chemicals found in olive oil on human research, which included an increase in Bacteroidetes and/or a decrease in the F/B ratio (Farràs et al., 2020).

Table 1. Changes in bacterial phyla (relative abundances (%)) throughout 24 h in vitro batch culture fermentations inoculated with human feces (n = 3 healthy donors) and administrated with fructo-oligosaccharides (FOS), Eco4%, and Eco0% as the substrates.

	Baseline			**Endpoint**								
Phylum				***FOS***			***Eco0%***			***Eco4%***		
Firmicutes	54.005	±	0.635^{b}	41.997	±	1.111^{a}	63.470	±	0.651^{c}	41.509	±	0.596^{a}
Bacteroidetes	33.997	±	0.741^{a}	23.957	±	0.870^{b}	17.418	±	0.422^{c}	33.264	±	0.529^{a}
Actinobacteria	7.537	±	0.613^{a}	27.832	±	1.232^{b}	6.338	±	0.738^{a}	16.696	±	1.058^{c}
Proteobacteria	1.762	±	0.193^{a}	3.577	±	0.544^{b}	11.628	±	1.344^{c}	5.571	±	0.798^{b}
Verrucomicrobia	1.775	±	0.218^{a}	1.175	±	0.128^{b}	0.207	±	0.065^{c}	1.910	±	0.478^{a}
Euryarchaeota	0.145	±	0.023^{a}	0.010	±	0.002^{c}	0.076	±	0.006^{b}	0.030	±	0.004^{c}
Fusobacteria	0.009	±	0.001^{a}	0.001	±	0.000^{a}	0.001	±	0.000^{a}	0.084	±	0.014^{b}
Synergistetes	0.011	±	0.002^{a}	0.001	±	0.000^{b}	0.007	±	0.002^{a}	0.001	±	0.000^{b}
Tenericutes	0.009	±	0.001^{a}	> 0.001	±	0.000^{b}	0.001	±	0.000^{a}	> 0.001	±	0.000^{b}
Crenarchaeota	0.001	±	0.000^{a}	> 0.001	±	0.000^{b}	> 0.001	±	0.000^{b}	0.001	±	0.000^{a}
Bacteria; Other	0.637	±	0.098^{a}	0.038	±	0.009^{c}	0.071	±	0.012^{b}	0.107	±	0.021^{b}
Archaea; Other	0.004	±	0.001^{a}	0.001	±	0.000^{a}	0.002	±	0.000^{a}	0.001	±	0.000^{a}
Unclassified	0.030	±	0.005^{a}	0.005	±	0.001^{b}	0.017	±	0.005^{a}	0.018	±	0.006^{a}
F/B	1.589	±	0.053*	1.753	±	0.017*	3.644	±	0.051§	1.247	±	0.012*

^{a,b,c} Letters or, *,§ Symbols indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$); ^{1} F/B = Firmicutes/Bacteroidetes. Samples were analyzed at 0 h (baseline) and 24 h. Values are means (%) with S.D.

At the lowest taxonomic level, 189 distinct bacterial OTUs were constructed and assigned (cutoffs 0.001%). Of these, 97 were identified at the baseline, while 54, 78, and 72 were identified at the endpoint of FOS, Eco0% and Eco4% fermentations, respectively. A dataset of 62 significant bacterial OTUs (Table S3) was generated after ANOVA ($p > 0.05$) and used to perform untargeted multivariate analysis by PCA. Successful discrimination of the variables among the substrates was achieved at the endpoint (Figure 1). In detail, major descriptors for FOS were *Bifidobacterium adolescentis*, *Bif. Bifidum*, *Akkermanisa muciniphila*, and *Roseburia faecis*. Those for Eco0% were the *Bacteroides cellulosyliticus*, *Dorea formicigerans*, and *Bilophila wadsworthia*. Those for Eco4% were *Megasphera elsdenii*, *Parabacteroides dista- sonis*, *Entorococcus durans*, *Bif. longum*, *Faecalibacterium prausnitzii*, *Lactobacillus plantarum*, *B. massiliensis*, *B. caccae*, and *B. uniformis*.

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The OTUs with the highest increases ($p < 0.05$) at the endpoint were *Bif. adolescentis*, *Bif. longum*, and *Lach. pectinoschiza* after FOS fer- mentation, and *M. elsdenii*, *Ent. durans*, *P. distansonis*, *B. massiliensis*, *L. plantarum*, and *Bif. longum* after Eco4% fermentation. In contrast, variables with maximum reductions ($p < 0.05$), either after FOS or Eco4%, were *F. prausnitzii*, *B. vulgatus*, *Ruminococcus gnavus*, *Citrobacter freundii*, *E. albertii*, and *Bil. wadsworthia*. Thus, from our results, even at the depth of the species level, it was possible to highlight the prebiotic potential of Eco4% that, similarly to FOS, fostered probiotic bacteria as well as beneficial bacteria, such as the SCFAs-producer *M. elsdenii* (Louis et al., 2014), MCFAs- and sphingolipids-producer *B. massiliensis* (Hiippala et al., 2020), succinate-producer *P. distasonis* (Wang et al., 2019), and competitive excluders *Lactobacillales* (Callaway et al., 2008), as *L. plantarum* (Heeney et al., 2019) and *E. durans* (Soltani et al., 2021). Moreover, Eco4% had the highest loads in beneficial SCFAs-producer *F. prausnitzii* (Dewulf et al., 2012) and fiber-degrading *B. caccae* (Nakajima et al., 2020). Fitting with the con- cept of prebiotics, Eco4% was even able to limit and contrast the growth of opportunistic (*Citrobacter freundii*) (Ganji et al., 2016) and close relative pathogenic species (*Escherichia albertii*) (Ooka et al., 2012), as well as that of species related to metabolic syndrome enterotypes, such as *R. gnavus* (Henke et al., 2019) and sulphate-producer *Bil. wadsworthia* (Natividad et al., 2018).

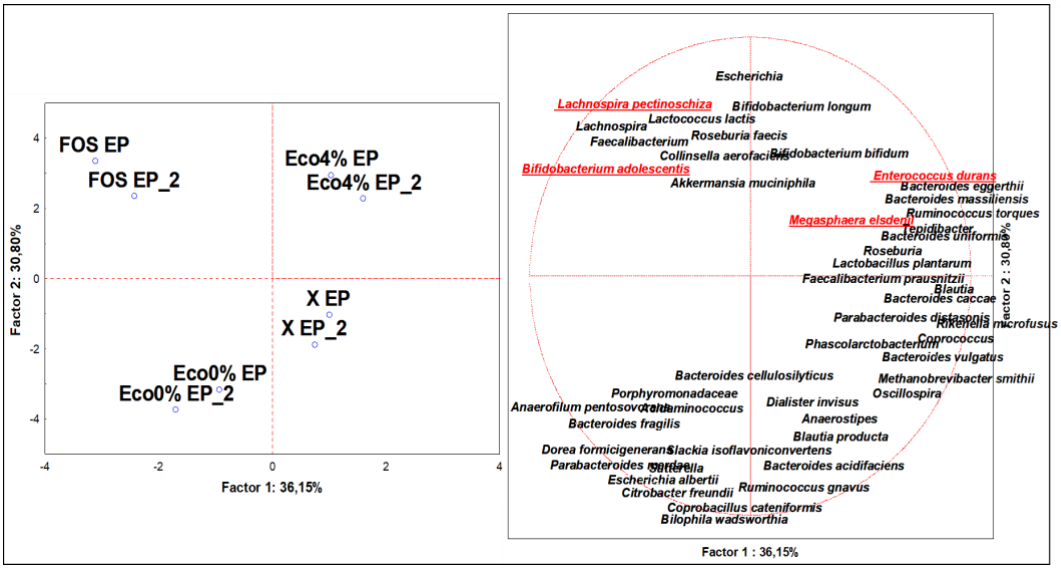

Figure 1. Principal component's analysis (PCA) of relative abundances (%) of significative (ANOVA $p < 0.05$) assigned Operational Taxonomic Units (OTUs) at the species level, after 24 h (EP = Endpoint; EP_2 = duplicate sample) of in vitro batch culture fermentations inoculated with human feces ($n = 3$ healthy donors) and administrated with FOS, Eco0%, and Eco4% as the substrates and a blank control (X). Variables in red font are the main descriptors of Eco4% or FOS cases.

4.3.4. Changes in Selected Fecal Bacterial Populations Measured with qPCR

Changes in *Eubacteria* kingdom, *Lactobacillales* order, *Bifidobacteriaceae*, *Enterobacteriaceae*, and *Clostridiaceae* families, and *E. coli* species were also assessed by qPCR (Table 2). At the early timepoint (5 h), no significant changes were found among all cases and bacterial targets ($p > 0.05$). At the intermediate timepoint (10 h), *Bifidobacteriaceae* and *Lactobacillales* significantly increased in numbers for FOS and Eco4% fermentations, while *Clostridiaceae* increased for Eco0% ($p < 0.05$). At the endpoint (24 h), almost all bacterial targets significantly changed in abundance ($p < 0.05$). For example, FOS, along with Eco4%, had increased numbers of total *Eubacteria*, *Bifidobacteriaceae*, and *Lactobacillales*. Eco4%, along with Eco0%, even recorded an increase in *Clostridiaceae*. Exclusively, Eco0% caused *Enterobacteriaceae* and *E. coli* surging. Besides this, Eco4% contributed to the significant reduction in *E. coli*. Our findings are consistent with those reported in the literature by comparable investigations using comparable colon model organisms (Connolly et al., 2012; Koutsos et al., 2017), and those of FOS and Eco4% are strictly restricted to the concept of prebiotics, which requires a substance to promote the growth of beneficial and probiotic bacteria (*Bifidobacteriaceae* and *Lactobacillales)* while simultaneously reducing that of opportunistic and pathogenic bacteria (*Enterobacteriaceae* and *E. coli*). The divergent shift found at the termination for *Clostridiaceae* was the only distinction between FOS and Eco4%. Similar traits have been described in other studies exploring the effects of olive oil on gut flora (Hidalgo et al., 2017).

Table 2. Changes in bacterial populations measured by qPCR on a RotorGene 6000 (Qiagen, Hilden, Germany) with the SYBR Green I chemistry, expressed as mean values in Log_{10} cells/mL.

	Time (h)	*Eubacteria*	*Bifidobacteriaceae*	*Lactobacillales*	*Enterobacteriaceae*	*Escherichia coli*	*Clostridiaceae*
FOS	0	9.16 ± 0.16^{a}	6.77 ± 0.11^{a}	7.36 ± 0.09^{a}	8.60 ± 0.07^{b}	4.08 ± 0.03^{ab}	7.15 ± 0.11^{a}
	5	9.32 ± 0.10^{a}	6.99 ± 0.12^{ab}	7.76 ± 0.10^{ab}	8.62 ± 0.13^{b}	4.40 ± 0.10^{b}	7.22 ± 0.04^{a}
	10	9.77 ± 0.09^{ab}	7.48 ± 0.09^{b}	8.31 ± 0.09^{b}	8.51 ± 0.02^{ab}	4.62 ± 0.07^{b}	7.67 ± 0.03^{ab}
	24	10.09 ± 0.28^{b}	8.81 ± 0.23^{c}	8.79 ± 0.11^{b}	8.05 ± 0.06^{a}	3.62 ± 0.07^{a}	7.34 ± 0.30^{a}
Eco 0%	0	9.12 ± 0.25^{a}	6.47 ± 0.08^{a}	7.11 ± 0.09^{a}	8.71 ± 0.08^{b}	4.00 ± 0.07^{ab}	7.11 ± 0.11^{a}
	5	9.00 ± 0.11^{a}	6.71 ± 0.09^{a}	7.65 ± 0.11^{ab}	8.91 ± 0.12^{bc}	4.40 ± 0.08^{b}	7.35 ± 0.11^{a}
	10	9.41 ± 0.26^{a}	6.68 ± 0.09^{a}	7.90 ± 0.14^{ab}	9.14 ± 0.11^{bc}	4.92 ± 0.11^{bc}	7.95 ± 0.21^{b}
	24	9.57 ± 0.07^{ab}	6.27 ± 0.08^{a}	7.71 ± 0.11^{ab}	9.44 ± 0.23^{c}	5.13 ± 0.21^{c}	8.10 ± 0.10^{b}
Eco 4%	0	9.02 ± 0.12^{a}	6.77 ± 0.10^{a}	7.24 ± 0.10^{a}	8.40 ± 0.06^{ab}	4.31 ± 0.07^{b}	7.01 ± 0.10^{a}
	5	9.22 ± 0.08^{a}	7.10 ± 0.10^{ab}	7.36 ± 0.11^{a}	8.62 ± 0.11^{b}	4.17 ± 0.17^{ab}	7.23 ± 0.20^{a}
	10	9.70 ± 0.09^{ab}	7.74 ± 0.09^{b}	7.98 ± 0.21^{b}	8.70 ± 0.08^{b}	4.22 ± 0.16^{ab}	7.47 ± 0.10^{ab}
	24	10.03 ± 0.20^{b}	8.55 ± 0.15^{c}	8.80 ± 0.14^{b}	9.16 ± 0.19^{bc}	3.92 ± 0.11^{a}	8.01 ± 0.19^{b}

^{a,b,c} Different letters among a bacterial target indicate significance by Tukey's HSD test ($p < 0.05$).

4.3.5. Volatilome Analysis through SPME GC/MS

Through SPME GC-MS, among 24 duplicated cases (n = 48), 161 identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). At the baseline, 92 were on average relatively quantified, and 120 were quantified over the course of the 24 hours of the experiment at various timepoints (Figure S4). A dataset of 49 significant molecules (ANOVA at $p < 0.05$) was created in order to describe the landscape of the volatilome. These molecules were then sorted and super-normalized by the chemical classes of VOCs, including organic acids, aldehydes, ketones, alcohols, and indoles. While multivariate analyses, such as untargeted Principal Component Analysis (PCA) and targeted MANOVA (p 0.01), were accomplished from each dataset of the other classes to address the specific contributions to VOCs production by the independent variables, organic acids and indoles are discussed in paragraph 3.7 as the main microbial metabolites related to prebiotic activity. To reveal the impact of compounds that are less volatile than others and may be underreported, as well as to avoid comparing one chemical class to another, super-normalization of the dataset was necessary (Nissen et al., 2020). Aldehydes are a result of microbial fermentation and lipid oxidation, as well as the transformation of ethanol (Malaguarnera et al., 2014). Some aldehydes, like Indole-3-aldehyde (Alexeev et al., 2018), are beneficial to health because they support cell homeostasis and microbiota eubiosis, while the majority are harmful because they are cytotoxic at low concentrations, like Acetaldehyde (Na et al., 2017). Distribution of cases on the plot, as determined by a PCA of 10 statistically significant aldehydes, distinguished fermentation with FOS and Eco4% from one another and from the baseline (Figure 2A). 2-Nonenal (E), which was mostly formed at the conclusion of the experiment (24 hours) ($p < 0.01$), was the key indicator of fermentation with Eco4%, whereas Benzeneacetaldehyde, Nonanal, and 2-Nonenal (E) were the main indicators of fermentation with FOS (butanal, 2-methyl) ($p < 0.01$). (Figure S5A, B). 2-Nonenal, (E) was reported to limit the growth of several intestinal pathogens at a very low concentration (Cho et al., 2004). It is conceivable that this resulted from the degradation of Palmitoleic acid (Mitro et al., 2012), which is one of the main fatty acids in olive oil. During colon fermentation, many ketones are produced; considering their bioactive attributes, some are desirable, such as the ketones bodies (Cabrera-Mulero et al., 2019), others, such as acetone, are unwanted, because they could be toxic for the host (Bradberry et al., 2007). 13 statistically significant ketones were distributed as cases on the plot by the PCA, which also distinguished the substrates from the baseline and one another (Figure 2B). Hexanone-5-methyl, 2-Butanone, and Acetophenone, which were primarily formed towards the endpoint, were descriptors of fermentation with Eco4% ($p < 0.01$). 2-Butanone, 4-hydroxy was the primary indicator of fermentation with FOS ($p < 0.01$),

whereas acetone was the primary indicator of fermentation with Eco0% ($p < 0.05$). (Figure S5C, D). Acetophenone merits consideration since it has antibacterial effects on several Gram-negative bacteria (Tran et al., 2020) and because its N-substitute derivatives have been suggested as a treatment for diabetes (Taslimi et al., 2020). In our experimental setting, when Eco4% is abundant, it most likely resulted via bacterial deconjugation of polyphenols. *Lactobacillales* (Carodna et al., 2013), a bacterial phylum indicated in such action, expanded after Eco4%. Alcohols are crucial components in the colon microbiota's fermentation of dietary polysaccharides (Oliphant et al., 2019). Separating fermentation with FOS and Eco4% from one another and from the baseline using PCA of 13 statistically significant alcohols distributed cases on the plot (Figure 2C). From our results, the contribution to alcohol production from the control samples remains undiscriminated ($p > 0.01$), while the descriptor of fermentation with FOS was mainly Ethyl alcohol ($p < 0.01$), and those for Eco4% were Phenethyl alcohol, 1-hexanol and 1-Pentanol, mainly produced at the late timepoints ($p < 0.01$) (Figure S5E, F). It is reported that 1-Pentanol is associated with the consumption of old grains, which have anti-inflammatory and prebiotic activity (Saa et al., 2014).

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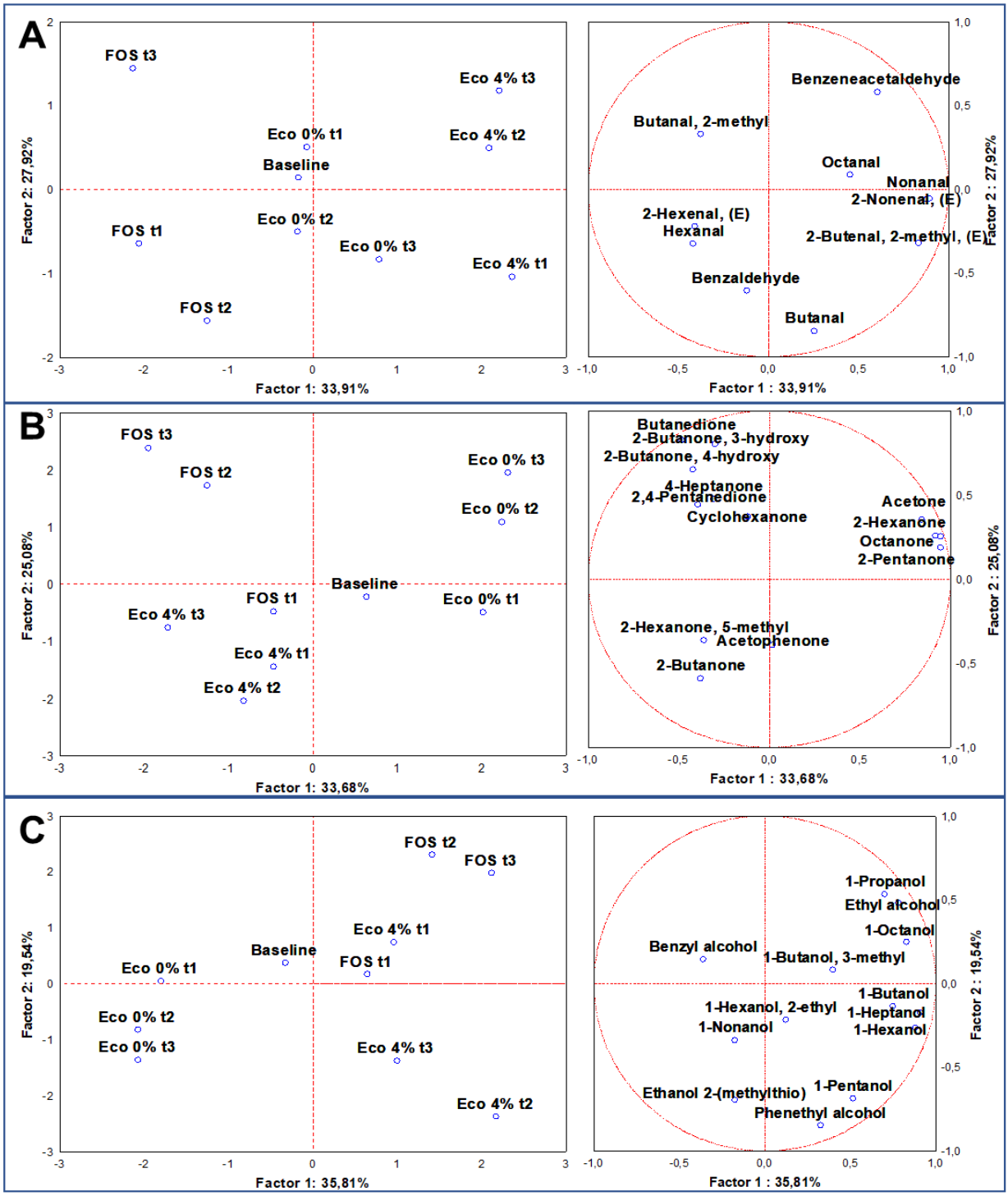

Figure 2. PCAs of the volatilome sorted by chemical classes of significative (ANOVA $p < 0.05$) VOCs, including the baseline and three different timepoints (t1 = 5 h; t2 = 10 h; t3 = 24 h). (**A**) = Aldehydes; (**B**) = Ketones; (**C**) = Alcohols. Left-side diagrams are for PCAs of cases, while right-side diagrams are for PCAs of variables.

4.3.6. Changes in Main Microbial Metabolites Related to Prebiotic Potential

To analyze the main changes in volatile microbial metabolites related to prebiotic potential, we considered the shift in loads from the baseline to the endpoint (24 h) of the fermentation of 13 selected VOCs with renowned bioactivity in humans (SCFAs, MCFAs, BCFAs, Indole and Skatole) as follows: (a) each single compound was normalized (mean centering method) within its dataset, which included cases from different type of sample; (b) the baseline dataset (Table S2) was then subtracted to the endpoint dataset; (c) post-hoc analysis was done to compare the sample productions of a single molecule (Tukey's HSD, $p < 0.05$). Short Chain Fatty Acids (SCFAs) are essential compounds for the host, the mucosa, and the colon microbiota. From our results (Figure 3A), SCFA concentration increased with FOS and Eco4%, while Acetic and Propanoic acid concentration decreased with Eco0%. The capacity to produce SCFAs was in the order FOS>ECO4%>ECO 0% ($p < 0.05$). Reduced intestinal cell homeostasis and decreased eubiosis of the gut microbiota are associated with decreased SCFA levels (Moens et al., 2019). The prebiotic activity of Eco4% could, therefore, be linked to its capacity to foster bacteria, deconstructing fibers and producing SCFAs. In fact, as was already said, we saw an increase in the good bacteria *Lactobacillus spp.*, *Bifidobacterium ssp.*, and *Enterococcus spp.*, which work diligently to produce SCFAs (Gibson et al., 2017). Given that previous studies have noted that these species are able to hydrolyze oleuropein, a polyphenol abundant in olive oil, to generate hydroxycortisol acetate (Farràs et al., 2020; Santos et al., 2012) it is possible that this action is caused by the PRF compounds of Eco4%. Medium-Chain Fatty Acids (MCFAs) are significant metabolic biomarkers of dysbiosis associated with Intestinal Bowel Disease (IBD) and have protective effects on glucose homeostasis after high-fat overeating and against insulin resistance (Lundsgaard et al., 2021). After fermentation with FOS or Eco4%, MCFA content increased in comparison to initial levels (Figure 3B and Table S2). After Eco0% fermentation, very little alterations were seen, suggesting that the Eco4% effect was likely more closely linked to changes in the colon microbiota than the bread's fatty acid composition. Except for Octanoic acid, whose production was 5.7 times more in ECO4% than in FOS ($p < 0.05$), FOS fermentation produced the greatest amounts of any MCFAs examined. Additionally, compared to its control, Eco0%, Eco4% produced 5.2 and 8.9-fold more Hexanoic and Heptanoic acids, respectively ($p < 0.05$). According to our prior research, Eco4% has the capacity to promote commensals like *Enterobacteriaceae* and *Bacteroides spp.* as well as *Bifidobacteriaceae*, which may explain the higher abundance of MCFAs seen in our study. During fiber fermentation, these three bacterial groups produced MCFA (Scarborough et al., 2019); Riviere et al., 2018). Branched-Chain Fatty Acids (BCFAs), including Propanoic acid, 2-methyl, Butanoic acid, 3-methyl, and Pentanoic acid, 3-methyl, are produced by the fermentation of microbial colon protein and may be stressful to the host (Aguirre et al., 2017).

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With the promoted objective employed to lower their concentration and improve health outcomes, BCFAs are frequently used as a biomarker of protein catabolism (Yao et al., 2016). Still, little is known about the impact of BCFAs on host health (Oliphant et al., 2019). What is undisputed, however, are the negative consequences of the pro-inflammatory and cytotoxic compounds yielded from the sulfur-containing, basic and aromatic amino acids (Oliphant et al., 2019). From our results, BCFAs (Figure 3C) increased with just Eco0%. Modest increments were seen for Propanoic acid, 2-methyl (Prop2M) and for Pentanoic acid, 3-methyl (Penta3M), when FOS and Eco4% were supplied, respectively. FOS and Eco4%, therefore, were able to reduce Butanoic acid, 3-methyl (Buty3M) similarly. In contrast to the surge for the three BCFAs produced by Eco0%, a decrease or increase driven by Eco4% may be observed, demonstrating how our food product is influencing the microbiota and encouraging the growth of the core microbiota that specializes in the fermentation of fibers rather than protein fermentation. According to (Diether et al., 2019), AA fermentation may have been hindered by the two experimental breads' differing ratios of fermentable carbohydrates and protein available to the microbiota. Since a similar situation was observed after FOS fermentation, another notch was added to the prebiotic potential of Eco4%. Two tryptophan catabolism byproducts, Indole and Skatole, result from the breakdown of the food's proteinaceous component. In addition to the host's metabolism of tryptophan, the local microbiota can directly convert tryptophan into indoles, from which a variety of derivatives are produced (Agus et al., 2018; Hendrikx et al., 2019). Although indole may also have positive effects, such as the reduction of inflammation indicators (Bansal et al., 2009), its bacterial production (*Clostridium spp*. and *Escherichia spp.)* and accumulation is hazardous for the host because it changes the mucosa's permeability and homeostasis (Roager et al., 2018). It can cause vascular disease and chronic renal disease after being converted into indoxyl sulphate in the liver (Wang et al., 2020; Hendrikx et al., 2019). In addition, tryptophan is decarboxylated by bacteria (*Bacteroides spp*. and *Clostridium spp*.) to create skatole (Indole,3-methyl), which triggers the release of pro-inflammatory cytokines (Roager et al., 2018). According to our findings (Figure 3D), the changes in Indole and Skatole concentration observed in the FOS and Eco4% fermentations over time relative to baseline were lower than those observed in the Eco0% fermentations. Similar to what happened for BCFAs, the prebiotic potential of Eco4% could be ascribed to PRF addition, which improved the proportions of fermentable protein and carbohydrates in the experimental bread, shaping the microbiota to the advantage of bacterial groups specialized in fibers more than protein fermentation.

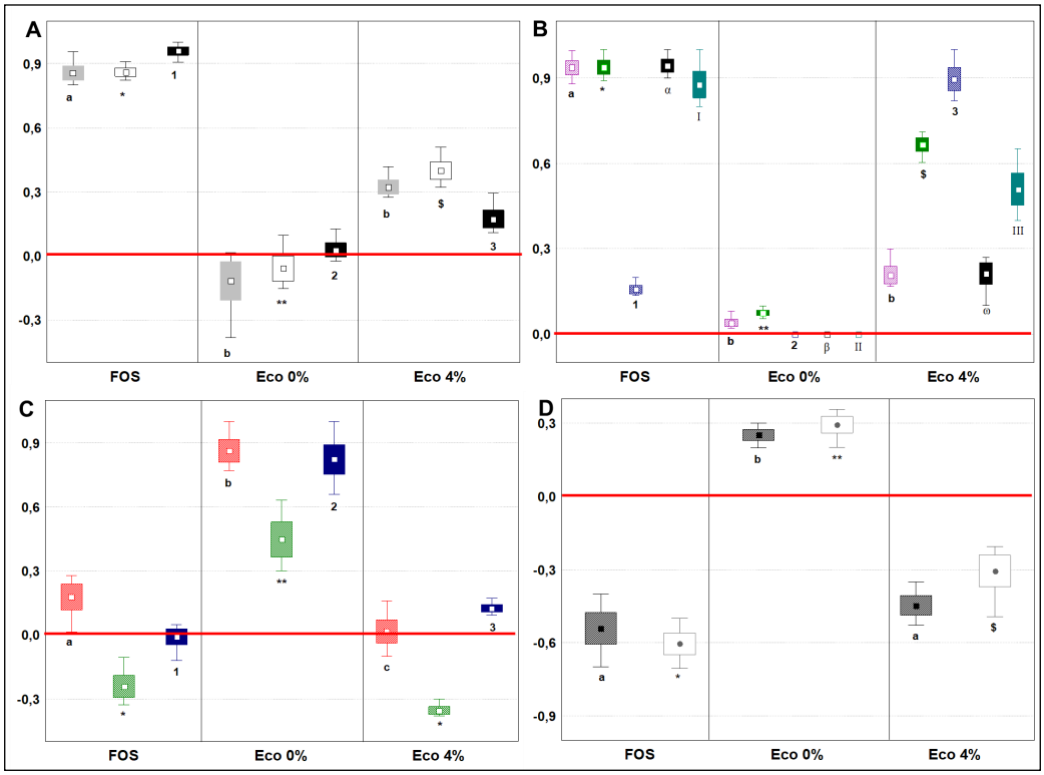

Figure 3. Endpoint changes in main microbial metabolites related to prebiotic activity, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/Kg are found in the Supplementary Material (Table S2). Changes were recorded after 24 h of in vitro batch colonic fermentations inoculated with human feces (n = 3 healthy donors) and administrated with FOS, Eco 0%, and Eco 4%. Samples were analyzed in duplicate from two independent experiments (n = 4). Box = mean; Rectangles = mean * S.D.; Whiskers = min and max values. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to Tukey's HSD test ($p < 0.05$). (**A**) Short-Chain Fatty Acids: gray plot = Acetic acid; white plot = Propanoic acid; black plot = Butanoic acid. (**B**) Medium-Chain fatty Acids: Fuchsia plot = Hexanoic acid; green plot = Heptanoic acid; blue stripes plot = Octanoic acid; black plot = Nonanoic; pale blue plot = n-Decanoic acid. (**C**) Branched-Chain Fatty Acids: red plot = Propanoic, 3-metyl acid; green plot = Butanoic, 3-methyl acid; blue plot = Pentanoic, 2-methyl acid. (**D**) Indoles: gray plot = Indole; white plot = Skatole.

4.3.7. Interomics Correlations among Metabolites Related to Prebiotic Potential and the Microbiota

Spearman Rank Correlations ($p < 0.05$), two-joining-way Heatmaps, and Pearson cluster analysis were performed by the comparison of two different normalized datasets, each derived from values of relative quantification (OTUs and VOCs) (Figure 4). The significance of correlations is reported in the Figure S6. From the Pearson dendrograms, three clusters were identified: the first two could strengthen the outputs on prebiotic potential presented over Eco4% fermentations, while the third includes less abundant OTUs and is supposedly less metabolically active in Eco4%. The first cluster included *Bif. adolescentis*, *M. elsdenii*, *Lach. pectinoshiza*, and *Colinsella aerofaciens*, and it was

positively correlated to SCFAs and MCFAs abundances, and inversely correlated to Pentanoic acid, 3-methyl and Skatole. What is known is that *M. elsdenii* produces butyrate from acetate or lactate produced from *Bif. adolescentis* (Moens et al., 2019). Lactate, which is not an SCFA, is also produced as a result of fermentation, but does not accumulate in the colon because it is used by several SCFA-producing bacteria (Louis et al., 2014), like *Lach. pectinoshiza*, and *Colinsella aerofaciens*. Another explicative correlation was found in cluster 2, where the reduction in indole content and BCFAs after Eco4% fermentation could be due to the recovered high loads in *E. durans*, while the reduction in skatole content was correlated with well-represented OTUs in Eco4% fermentations, such as *F. prausnitzii* and *A. muciniphila*. Moreover, these three species, along with *P. distasonis* (cluster 1), were positively correlated with octanoic acid that we found after fermentations that were richer in Eco4% than FOS. The increased abundances in MCFAs were correlated in cluster 2 to more commensal *Enterobacteriaceae*, *Bif. longum*, and *Bif. bifidum*, as seen after FOS or Eco4% fermentations, and as explained by other authors [Scarborough et al., 2019; Riviere et al., 2018). Eco4% and FOS were even able to diminish the population of opportunistic *Enterobacteriaceae* and *Desulfovibrionaceae*, as well as the production of indoles and BCFAs; in fact, we found a positive correlation among *Cit. freundii*, *E. albertii* and *Bil. wadsworthia* (cluster 3) with the production of those detrimental compounds.

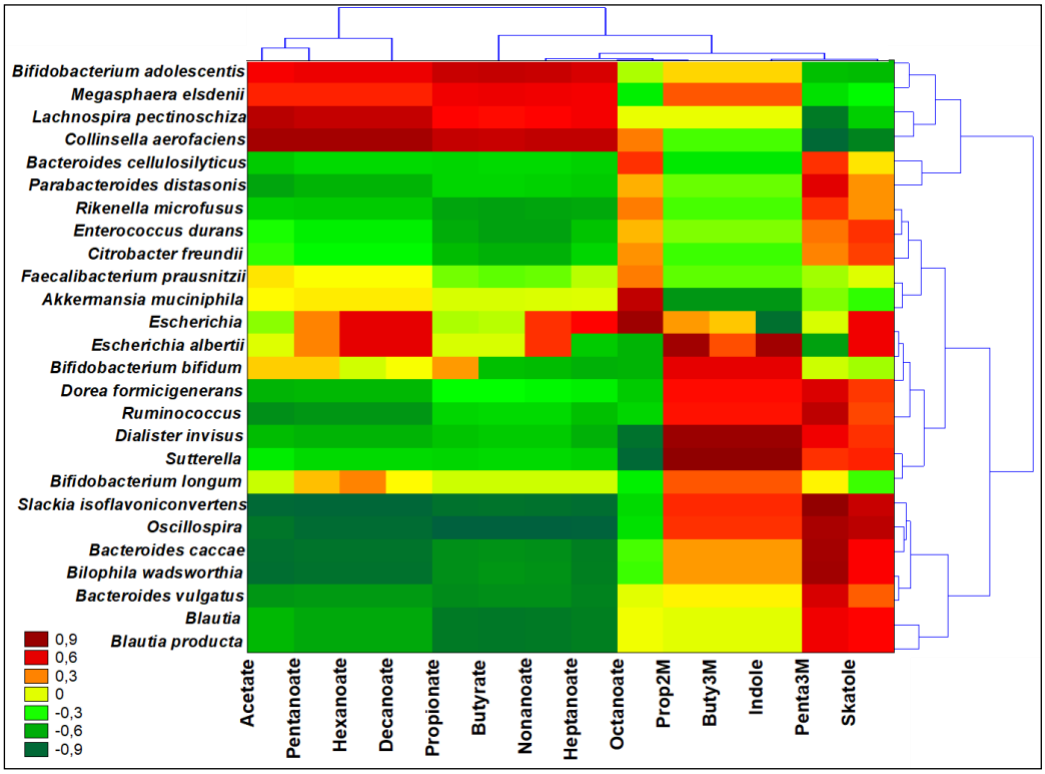

Figure 4. Interomics, Spearman Rank Correlations among main microbial metabolites from the volatilome and ANOVA significant ($p < 0.05$) species OTUs from the microbiota. Prop2M = Propanoic acid, 2-metyl; Buty3M = Butanoic acid, 3-methyl; Penta3M = Pentanoic acid, 3-methyl.

Leftside dendrogram identifies by Pearson analysis three major different clusters among bacterial species. Significance of correlations are provided as supplementary material (Figure S6).

4.4. Conclusions

Our investigation provided conclusive proof that Eco4% bread has prebiotic potential attributable to PRF addition based on the favorable results obtained by several prebiotics indicators. In fact, Eco4% had no effect on eubiosis and did not cause dysbiosis, keeping the F/B ratio in equilibrium and the microbiota's alpha diversity constant throughout the fermentation process. Other encouraging signs included increased SCFA and MCFA production as well as a decline in the abundance of dangerous BCFAs like indole and skatole. Additionally, following Eco4%, there was a drop in opportunistic or pathogenic species and an increase in probiotic or helpful organisms. A decrease in species linked to proteolytic fermentation was observed after Eco4%, while an increase in species linked to fiber fermentation was observed. Our findings were supported by a logical and understandable multivariate statistical technique that combined information from microbial genomes and metabolomics to create an interomic display that clearly illustrates the causes and consequences brought on by a specific fiber with prebiotic potential. The presented study used MICODE, a reliable and adaptable *in vitro* model, which was evaluated by a quality control check of various issues, including the presence of *Archea* species throughout the fermentation period, the ability of FOS to foster probiotics, the of similar observed OTUs in the system, as well as the rare species seen by Good's index, and, lastly, taking into account the volatilome, there were several stool-derived compounds kept at the same retention time. Even if *in vivo* animal models or diet-intervention studies should be used to fully elucidate the prebiotic potential of Eco4%, as well as to address specific host benefits, the recipient results we have presented are target-effective and should have robustness for pre-clinical applications.

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4.6. Supplementary materials

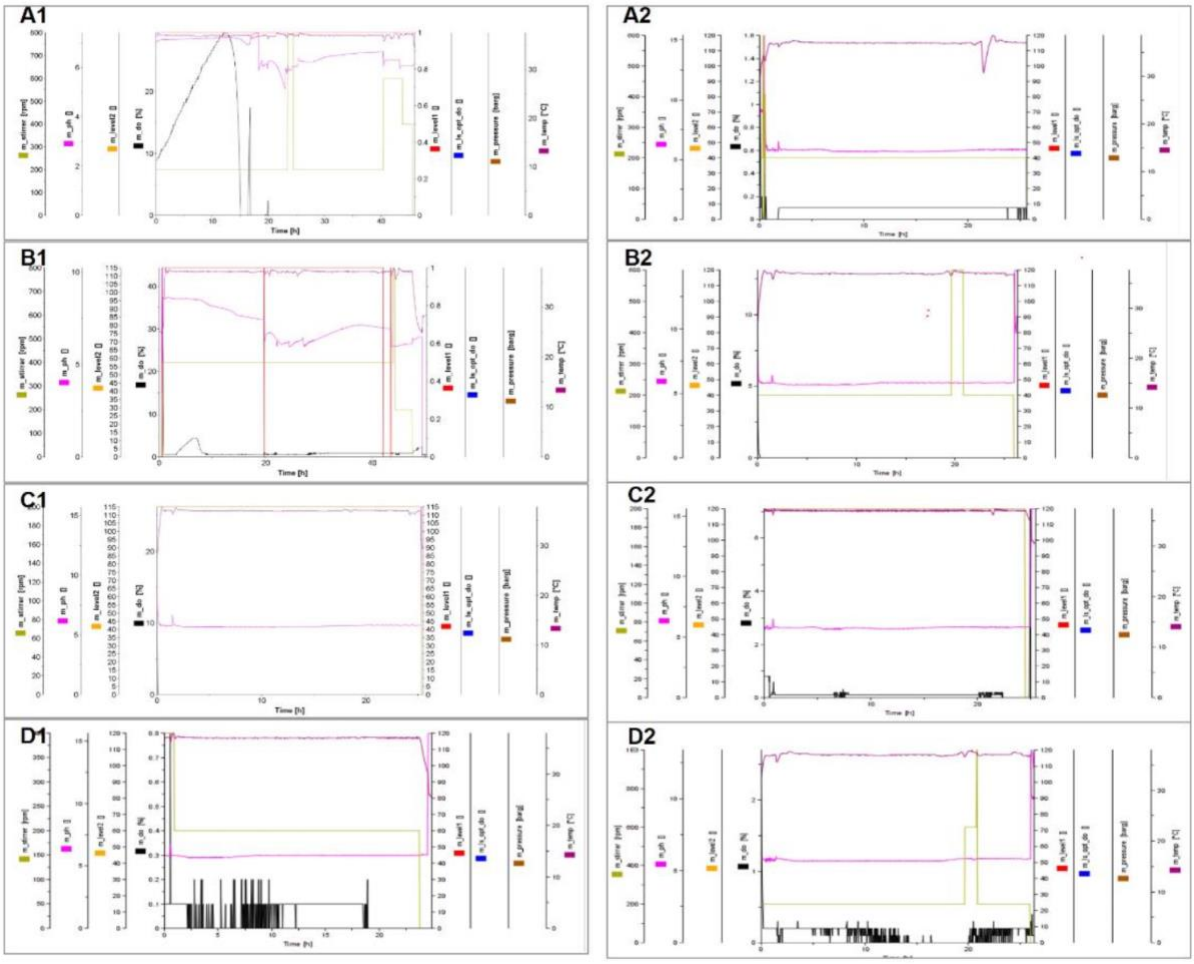

Figure S1. Plots of parameters trends of bioreactors, during colonic fermentations. A1 - A2: duplicate experiments on MICODE bioreactor with FOS; B1 - B2: duplicate experiments on MICODE bioreactor with Eco0%; C1 - C2: duplicate experiments on MICODE bioreactor with Eco4%; D1 - D2: duplicate experiments on MICODE bioreactor without supplements.

Table S1. Primers pair sequences for PCR-qPCR (Lane et al., 1992; Bartosch et al., 2004; Walter et al., 2001; Masco et al., 2004; Zhou et al., 1994; Goldberg et al., 2013).

Group	Target	Sequence 3'-5'	Bp	Reference
Eubacteria	V3-V4 16 S	Eub518R: ATTACCGCGGCTGCTGG Eub338R: ACTCCTACGGGAGGCAG	147	Lane *et al*, 1991
Enterobacteriaceae	V3-V4 16 S	Enterobac-f: TGCCGTAACTTCGGGAG Enterobac-r: TCAAGGACCAGTGTTCAG	450	Bartosh *et al*, 2004
Lactobacillales	V3-V4 16 S	F-Lac: GCAGCAGTAGGGAATCT R-Lac: GCATTYCACCGCTACACA	340	Walter *et al*, 2001
Bifidobacteriaceae	RecA	RecAf: CGTYTCBCAGCCGGAYA RecAr: CCARVGCRCCGGTCATC	220	Masco *et al*., 2006
E. coli	FtsZ	EcFtsZf: GGTATCCTGACCGTTGCT EcFtszr: ATACCTCGGCCCAGAACT	250	Zhou e Helmstetter, 1994
Clostridiaceae	V3-V4 16 S	ClosIV-f: TTAACACAATAAGTWATC ClosIV-r: ACCTTCCTCCGTTTTGTC	400	Goldberg *et al*., 2013

Table S2. Quantification of main microbial VOCs related to prebiotic activity at the baseline.

VOCs	mg/Kg ± SD
Acetic acid	0.465 ± 0.353
Propanoic acid	0.210 ± 0.161
Butanoic acid	0.700 ± 0.090
Propanoic acid, 2-methyl	traces
Butanoic acid, 3-methyl	0.434 ± 0.028
Pentanoic acid, 3-methyl	0.031 ± 0.007
Pentanoic acid	0.689 ± 0.122
Hexanoic acid	0.046 ± 0.030
Heptanoic acid	traces
Octanoic acid	traces
Nonanoic acid	< 0.01
n-Decanoic acid	< 0.01
Indole	3.879 ± 0.963
Skatole	0.408 ± 0.063

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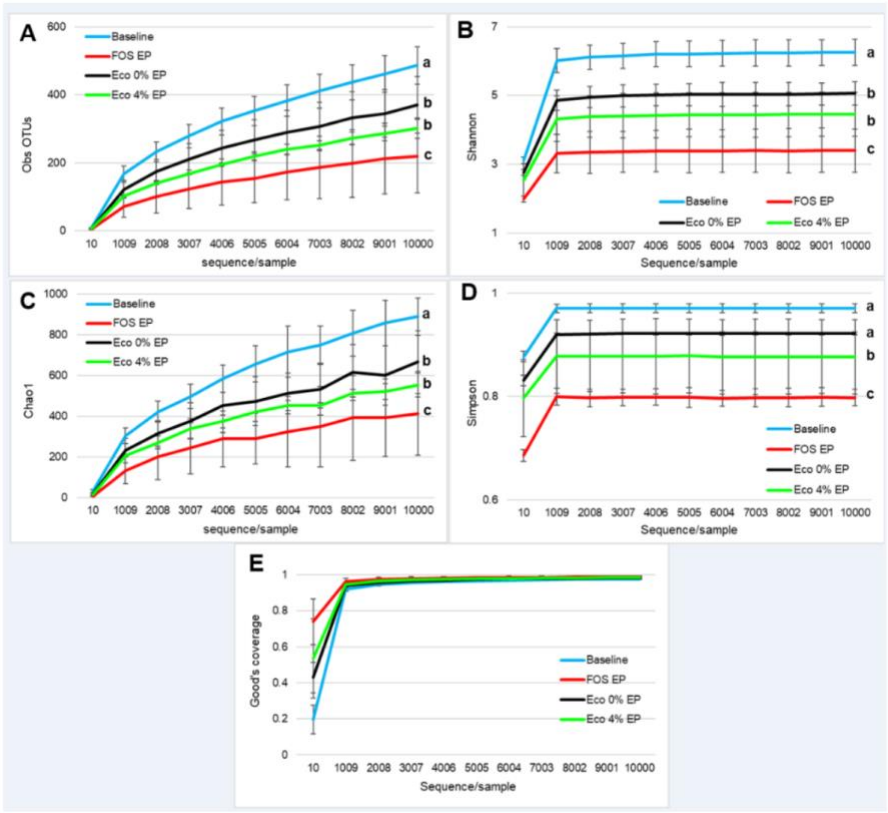

Figure S2. Plots of Alpha Diversity indices. A = Observed OTU index for microbiota richness; B = Shannon index for microbiota evenness; C = Chao 1 index for microbiota abundance; D = Simpson index for microbiota dominance; E = Good's index for microbiota rarity. Different letters indicate significance (*t*-student $p < 0.05$) within a plot.

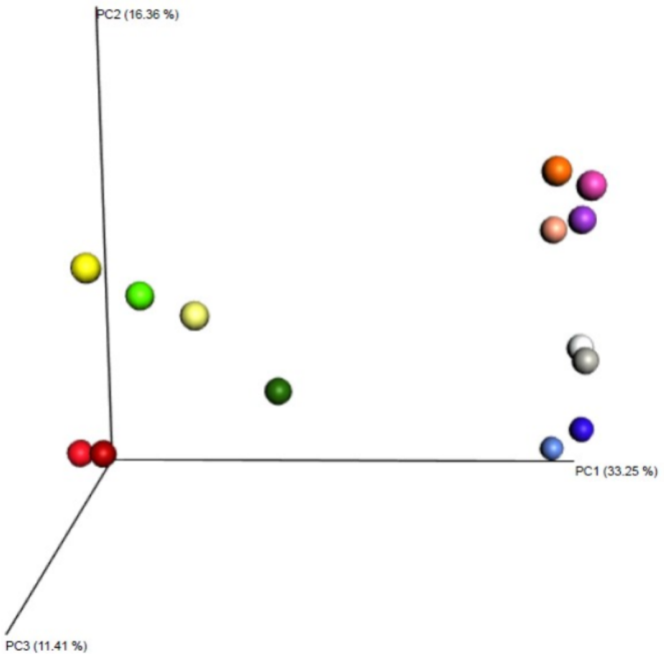

Figure S3. Bray Curtis PCoA of Beta Diversity. Fuchsia and magenta dots = duplicates of stool microbiota from donor 1; pink and violet = duplicates of stool microbiota from donor 2; blue and pale blue = duplicates of stool microbiota from donor 3; gray and pale gray = duplicates of fecal slurry (pool of the three stools); yellow and pale yellow = duplicates of effluents from FOS fermentations; green and lime = duplicates of effluents from Eco4% fermentations; orange and red = duplicates of effluents from Eco0% fermentations.

Table S3. Relative quantification of OTUs assigned at the species level.

Assigned OTUs	**Baseline**	**FOS 24 h**	**Eco0% 24 h**	**Eco4% 24 h**
Acidaminococcus;s__	0.0119^{a}	0.0000	17.0160^{b}	0.0000
Adlercreutzia;s__	0.0727^{a}	0.0165^{b}	0.0262^{b}	0.0259^{b}
Akkermansia;s__muciniphila	1.7751^{a}	2.6454^{a}	0.2026^{b}	1.8068^{a}
Anaerofilum;s__pentosovorans	0.0705^{a}	0.1974^{b}	0.2436^{b}	0.3538^{bc}
Anaerostipes;s__	0.2257^{a}	0.0000	0.0865^{b}	0.0155^{b}
*Bacteroides;*Other	1.2065^{a}	2.0981^{a}	7.3804^{b}	2.6273^{a}
Bacteroides;s__acidifaciens	0.1052^{a}	0.1691^{a}	0.4007^{bc}	0.7826^{c}
Bacteroides;s__caccae	0.6217	0.5140	0.6421	0.4564
Bacteroides;s__cellulosilyticus	0.2279^{a}	0.1487^{a}	7.2420^{b}	0.6635^{a}
Bacteroides;s__eggerthii	0.7270^{a}	0.2040^{b}	0.0228^{c}	0.0181^{c}
Bacteroides;s__fragilis	0.0250^{a}	1.0766^{b}	0.1252^{a}	1.2640^{b}
Bacteroides;s__massiliensis	0.4731^{a}	0.3567^{a}	0.3017^{a}	3.6914^{b}
Bacteroides;s__thetaiotaomicron	0.2702^{a}	0.2363^{a}	1.6632^{b}	2.5583
Bacteroides;s__uniformis	1.7512^{a}	0.3685^{b}	1.1851^{a}	3.8950^{c}
Bacteroides;s__ vulgatus	8.1140^{a}	2.1840^{b}	2.9667^{b}	3.9648^{b}
Bifidobacterium;s__adolescentis	3.0067^{a}	32.1257^{b}	2.1716^{a}	6.8630^{c}
Bifidobacterium;s__bifidum	0.7877^{a}	3.8160^{b}	0.7821^{a}	0.6489^{a}
Bifidobacterium;s__longum	1.3093^{a}	4.2386^{b}	2.2097^{b}	4.8900^{b}
Bilophila;s__wadsworthia	0.7862^{a}	0.0562^{b}	0.9735^{a}	0.3262^{a}
Blautia;s__	8.2039^{a}	0.1336^{b}	0.3358^{b}	0.1734^{b}
Blautia;s__obeum	2.5856^{a}	0.0526^{b}	0.2004^{b}	0.0742^{b}
Blautia;s__producta	0.2886^{a}	0.0000	0.0250^{b}	0.1363^{a}
Butyricimonas;s__	0.1562^{a}	0.0125^{b}	0.2163^{a}	0.1199^{a}
Citrobacter;s__freundii	0.7191^{a}	0.0010^{b}	0.0050^{b}	0.0289^{b}
Collinsella;s__aerofaciens	1.5353^{a}	2.2883^{a}	0.2425^{b}	0.4858^{b}
*Coprobacillaceae;*Other	0.2203^{a}	0.0099^{b}	0.0387^{b}	0.0242^{b}
Coprobacillus;s__cateniformis	0.0380^{a}	0.0138^{a}	0.5043^{b}	0.3727^{b}
Coprococcus;s__	6.9756^{a}	0.0270^{b}	0.2561^{b}	0.2563^{b}
*Coriobacteriaceae;*Other	1.0774^{a}	0.0592^{b}	0.2846^{b}	0.1484^{b}
Desulfovibrio;s__	0.5121^{a}	0.1053^{a}	0.8387^{b}	0.1631^{a}
Dialister;s__invisus	2.8362^{a}	0.0000	1.1813^{b}	0.9025^{b}
Dorea;s__formicigenerans	0.6478^{a}	0.3949^{a}	6.7692^{b}	1.3503^{a}
Enterococcus;s__durans	0.0174^{a}	5.4146^{b}	2.9246^{b}	7.5645^{b}
Escherichia;s__	0.4752^{a}	6.3164^{b}	9.6857^{b}	4.0286^{b}
Escherichia;s__albertii	0.6330^{a}	0.0230^{b}	1.0889^{a}	0.1430^{a}
Faecalibacterium;s__prausnitzii	8.7735^{a}	3.1281^{b}	0.0455^{c}	4.8725^{b}
Faecalibacterium;s__	0.2289^{a}	0.4607^{a}	0.1662^{a}	0.0216^{b}

Klebsiella;s__variicola	0.0043[a]	0.0000	0.0057[a]	0.0052[a]
Lachnospira;s__	0.9570[a]	0.7226[a]	0.1366[b]	0.1070[b]
Lachnospira;s__pectinoschiza	0.1628[a]	2.4107[b]	0.0205[c]	0.0069[c]
*Lachnospiraceae;*Other	0.2919[a]	0.0046[b]	0.1617[a]	0.6911[a]
Lactobacillus;s__plantarum	0.0000	2.2958[a]	0.0091[b]	4.1990[a]
Lactococcus;s__lactis	0.2930[a]	1.1955[a]	0.0023[b]	0.0035[b]
Megasphaera;s__elsdenii	1.8218[a]	12.3391[b]	9.0260[b]	16.2232[b]
Methanobrevibacter;s__smithii	0.6879[a]	0.0526[b]	0.2172[a]	0.2502[a]
Oscillospira;s__	2.1646[a]	0.3225[b]	0.8800[b]	0.3857[b]
Parabacteroides;s__	0.3917[a]	0.0816[a]	3.8454[b]	2.2278[b]
Parabacteroides;s__distasonis	4.4106[a]	0.5278[b]	3.0544[a]	13.2508[c]
Parabacteroides;s__merdae	0.1345	0.0000	0.0000	0.0000
Phascolarctobacterium;s__	0.0998[a]	0.0224[b]	0.0603[a]	0.1415[a]
*Porphyromonadaceae;*Other;Other	0.0000	0.0724[a]	0.6887[b]	0.3201[b]
Rikenella;s__microfusus	2.7842[a]	0.1856[b]	0.8504[b]	1.2468[b]
Roseburia;s__	2.6909[a]	0.1316[b]	0.0273[b]	0.0544[b]
Roseburia;s__faecis	0.1367[a]	2.3558[b]	0.0137[c]	0.0017[d]
*Ruminococcus;*Other	10.6419[a]	1.0395[b]	0.5007[b]	0.0000
Ruminococcus;s__	2.8015[a]	0.2896[b]	2.2461[a]	0.4823[b]
Ruminococcus;s__callidus	1.0102[a]	0.0000	0.0011[b]	0.0035[b]
Ruminococcus;s__gnavus	3.1470[a]	0.1777[b]	0.1776[b]	0.2457[b]
Ruminococcus;s__torques	0.7009[a]	0.0000	0.0091[b]	0.0052[b]
Slackia;s__isoflavoniconvertens	0.2040[a]	0.0243[b]	0.3700[a]	0.1320[a]
Sutterella;s__	0.4720[a]	0.6199[a]	1.1236[b]	0.3702[a]
Tepidibacter;s__	1.4843[a]	0.0816[b]	0.1685[b]	0.0682[b]

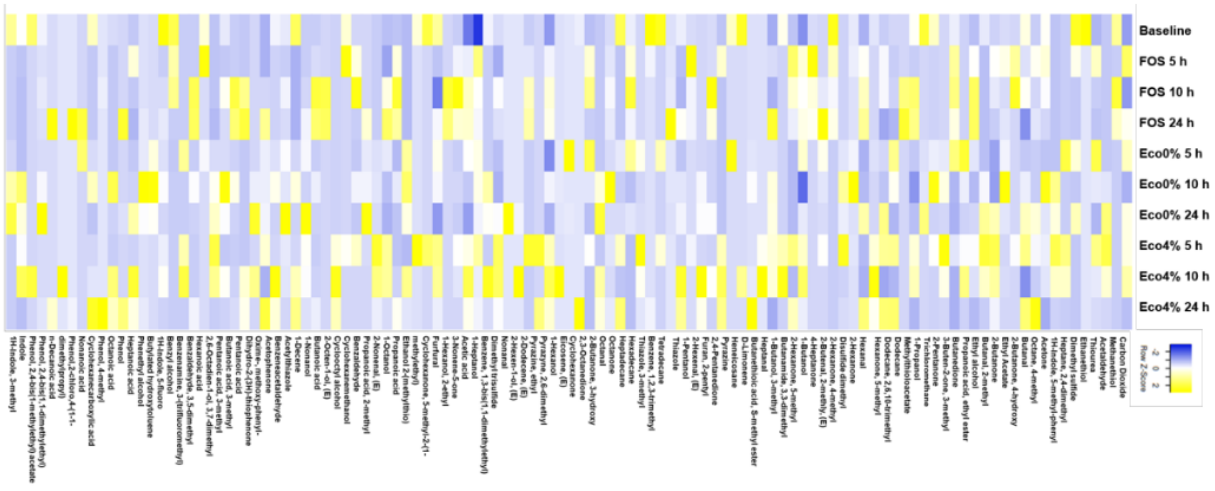

Figure S4. Quantification heatmap of total VOCs.

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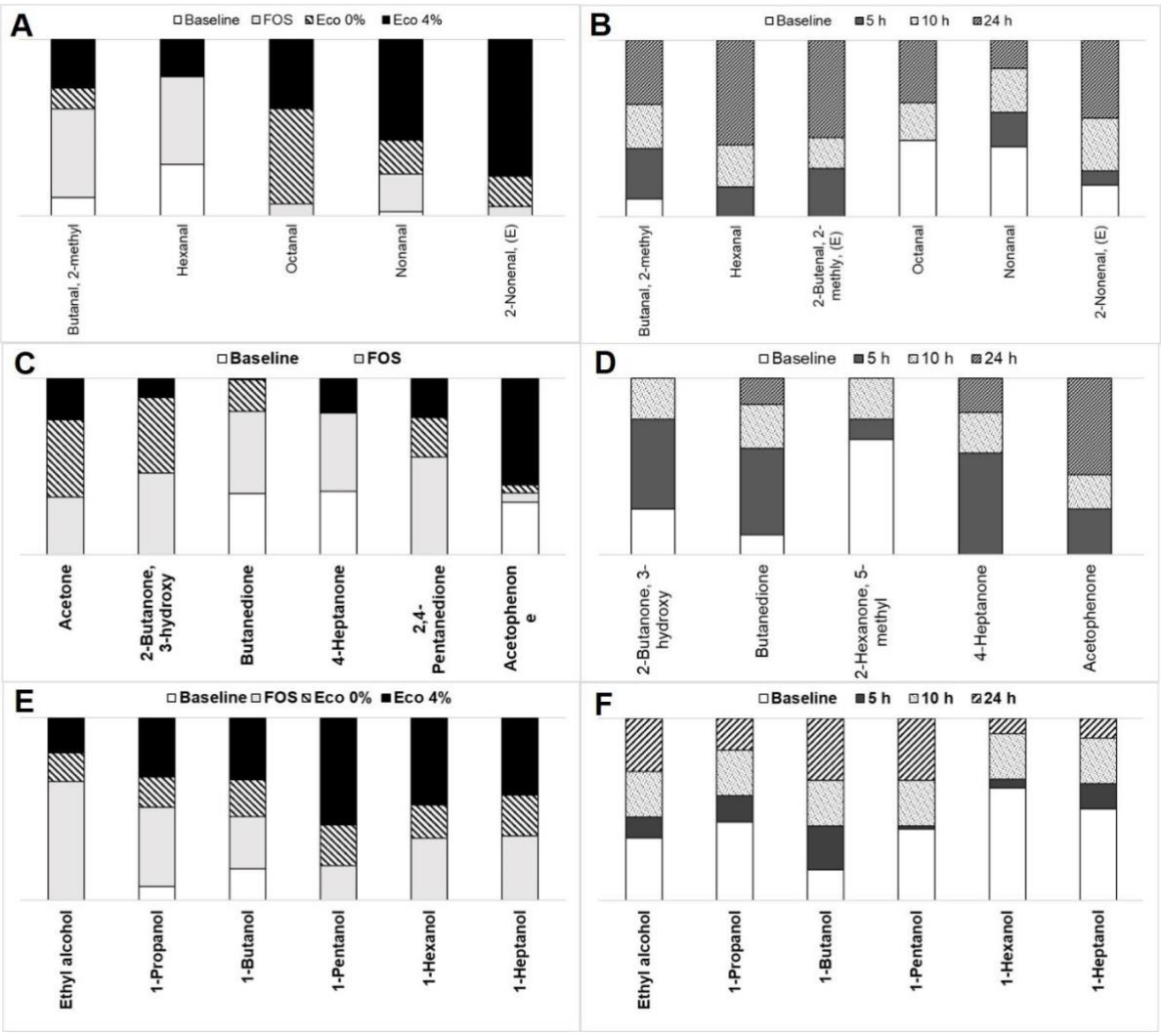

Figure S5. MANOVA plots by categorical descriptors for the volatilome

Spearman Rank Order Correlations (Spreadsheet34)
MD pairwise deleted
Marked correlations are significant at p <,05000

Variable	Acetic acid	Propanoic acid	Butanoic acid	Propanoic acid, 2-methyl	Butanoic acid, 3-methyl	Pentanoic acid, 3-methyl	Pentanoic acid	Hexanoic acid	Heptanoic acid	Octanoic acid	Nonanoic acid	n-Decanoic acid	Indole	1H-Indole, 3-methyl
Bifidobacterium;s__adolescentis	0,535714	0,689286	0,707143	0,157143	0,157143	-0,542857	0,564286	0,564286	0,648750	-0,150000	0,697051	0,564286	0,157143	-0,560714
Bifidobacterium;s__bifidum	-0,721429	-0,550000	-0,542857	0,596429	0,596429	0,778571	-0,725000	-0,725000	-0,591402	-0,582143	-0,555854	-0,725000	0,596429	0,539286
Bifidobacterium;s__longum	-0,882143	-0,782143	-0,775000	0,364286	0,364286	0,903571	-0,885714	-0,885714	-0,806457	-0,385714	-0,788204	-0,885714	0,364286	0,675000
Bacteroides;s__caccae	-0,860714	-0,710714	-0,689286	0,260714	0,260714	0,846429	-0,832143	-0,832143	-0,763446	-0,260714	-0,700626	-0,832143	0,260714	0,517857
Bacteroides;s__cellulosilyticus	-0,500000	-0,464286	-0,457143	-0,400000	-0,400000	0,435714	-0,457143	-0,457143	-0,483874	0,435714	-0,453977	-0,457143	-0,400000	0,132143
Bacteroides;s__vulgatus	-0,675000	-0,717857	-0,710714	0,110714	0,110714	0,646429	-0,671429	-0,671429	-0,745525	-0,010714	-0,702413	-0,671429	0,110714	0,360714
Parabacteroides;s__distasonis	-0,625000	-0,475000	-0,467857	-0,214286	-0,214286	0,603571	-0,582143	-0,582143	-0,505380	0,221429	-0,478999	-0,582143	-0,214286	0,264286
Rikenella;s__microfusus	-0,489286	-0,635714	-0,639286	-0,260714	-0,260714	0,432143	-0,500000	-0,500000	-0,620076	0,303571	-0,627346	-0,500000	-0,260714	0,271429
Enterococcus;s__durans	-0,310714	-0,610714	-0,646429	-0,192857	-0,192857	0,317857	-0,382143	-0,382143	-0,526885	0,207143	-0,639857	-0,382143	-0,192857	0,432143
Blautia;s__	-0,575000	-0,789286	-0,803571	-0,007143	-0,007143	0,553571	-0,617857	-0,617857	-0,752693	0,042857	-0,793566	-0,617857	-0,007143	0,507143
Dorea;s__formicigenerans	-0,578571	-0,335714	-0,328571	0,489286	0,489286	0,635714	-0,564286	-0,564286	-0,376347	-0,510714	-0,350313	-0,564286	0,489286	0,417857
Lachnospira;s__pectinoschiza	0,760714	0,503571	0,496429	0,021429	0,021429	-0,789286	0,710714	0,710714	0,544806	0,014286	0,504022	0,710714	0,021429	-0,492857
Ruminococcus;s__	-0,707143	-0,471429	-0,450000	0,485714	0,485714	0,728571	-0,678571	-0,678571	-0,541222	-0,464286	-0,461126	-0,678571	0,485714	0,389286
Faecalibacterium;s__prausnitzii	0,132143	-0,203571	-0,232143	-0,225000	-0,225000	-0,160714	0,078571	0,078571	-0,136202	0,310714	-0,212690	0,078571	-0,225000	-0,021429
Oscillospira;s__	-0,817857	-0,957143	-0,960714	0,435714	0,435714	0,825000	-0,875000	-0,875000	-0,942658	-0,428571	-0,957999	-0,875000	0,435714	0,750000
Dialister;s__invisus	-0,553571	-0,535714	-0,510714	0,885714	0,885714	0,560714	-0,582143	-0,582143	-0,591402	-0,850000	-0,507596	-0,582143	0,885714	0,432143
Megasphaera;s__elsdenii	0,457143	0,557143	0,564286	0,364286	0,364286	-0,435714	0,453571	0,453571	0,537638	-0,385714	0,552279	0,453571	0,364286	-0,342857
Collinsella;s__aerofaciens	0,850000	0,703571	0,700000	-0,260714	-0,260714	-0,907143	0,839286	0,839286	0,724019	0,303571	0,713137	0,839286	-0,260714	-0,746429
Slackia;s__isoflavoniconvertens	-0,907143	-0,839286	-0,828571	0,439286	0,439286	0,921429	-0,917857	-0,917857	-0,867389	-0,453571	-0,838249	-0,917857	0,439286	0,700000
Sutterella;s__	-0,392857	-0,464286	-0,457143	0,939286	0,939286	0,435714	-0,457143	-0,457143	-0,483874	-0,903571	-0,453977	-0,457143	0,939286	0,453571
Bilophila;s__wadsworthia	-0,867857	-0,703571	-0,682143	0,253571	0,253571	0,853571	-0,839286	-0,839286	-0,756277	-0,267857	-0,693477	-0,839286	0,253571	0,525000
Citrobacter;s__freundii	-0,285714	-0,553571	-0,592857	-0,267857	-0,267857	0,300000	-0,350000	-0,350000	-0,465953	0,275000	-0,589813	-0,350000	-0,267857	0,407143
Escherichia;s__	0,582143	0,446429	0,425000	-0,857143	-0,857143	-0,646429	0,596429	0,596429	0,501795	0,864286	0,428955	0,596429	-0,857143	-0,557143
Escherichia;s__albertii	0,582143	0,446429	0,425000	-0,857143	-0,857143	-0,646429	0,596429	0,596429	0,501795	0,864286	0,428955	0,596429	-0,857143	-0,557143
Akkermansia;s__muciniphila	0,096429	-0,039286	-0,046429	-0,675000	-0,675000	-0,189286	0,117857	0,117857	-0,014337	0,725000	-0,032172	0,117857	-0,675000	-0,282143

Figure S6. Significance of Spearman rank correlations

5. CASE STUDY 2: Bread fortified by spirulina

The study presented below consisted of two sections:

- the first (**5.1.**) aimed at evaluating the effect of the formulation and fermentation process on gluten-free bread fortified with spirulina
- the second (**5.2.**) aimed at evaluating in vitro the effect of the aforementioned bread on the human intestinal microbiota

5.1. Effect of formulations and fermentation processes on volatile organic compounds and prebiotic potential of gluten-free bread fortified by spirulina (*Arthrospira platensis*)

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5.1.1. Introduction

Celiac disease (CD) is an autoimmune disease triggered by the ingestion of gluten present in wheat, barley, and rye in genetically predisposed individuals. The prevalence of celiac disease in the general population is 1%, with regional differences (Leonard et al., 2017), and its management requires exclusion of dietary gluten and the substitution of gluten-containing products with gluten-free (GF) products. The manufacturing of GF products is challenging not only from an organoleptic but also from a nutritional point of view. GF products are often nutritionally less adequate than standard products in view of their low protein and high fat, sugar, and salt content (Allen et al., 2018).

Gluten-free (GF) product development presents major challenges for the food industry in terms of organoleptic, technological, and nutritional characteristics. The GF food market is continuously growing, with estimated market share sales worldwide of 18% gluten-free pasta in 2022, with an annual growth rate of 7.4% (Hedin et al., 2016). Nowadays, target audience for GF foods stretches beyond coeliac sufferers. In 2015, only 9% of US gluten-free consumers followed a GF diet due to celiac disease, while others were adopting a GF lifestyle because it made them feel healthier (12%) or they wanted to lose weight (7%) (NSF, 2015).

In response to consumers needs, more and more gluten- free products, such as bread, have appeared on the market. However, these products often do not satisfy the nutritional deficiencies of these consumers in terms of dietary fiber, vitamins (B12, D), and minerals (iron, calcium, zinc) (Thompson et al., 2005). Furthermore, consumers consider a GF diet hard to follow due to low availability, lack of variety, texture problems, poor palatability, and high prices of the GF products (Nascimento et al., 2014). Moreover, GF products, especially the bakery ones are poor in protein content and a protein implementation is common (Missbach et al., 2015). However, the effects of protein fortification by different flours as the protein source on the food quality need to be clarified.

CASE STUDY 2: Bread fortified by spirulina

Arthrospira platensis (spirulina) powder/flour (FA) represents a potential ingredient for GF bakery products. This microalgae presents a high protein content, up to 70% dry weight (Plaza et al., 2009). Its amino acid composition has a great significance not only because *A. platensis* possesses all of the essential amino acids, but also because these amino acids have a great bio-availability (Plaza et al., 2009). The carbohydrates of A. platensis constitute approximately 15% of the dry matter. The major carbohydrates are polysaccharides. Among the monomeric forms, glucose, galactose, ribose, and mannose are preferentially found (Plaza et al., 2009). On the other hand, its lipid fraction accounts for about 5% of its dry weight (Plaza et al., 2009). *A. platensis* is rich in polyunsaturated fatty acids, carotenoids, vitamins, minerals, phenolic compounds, and bioactive molecules (Plaza et al., 2009). *A. platensis* also shows various activities of pharmacological interest, such as antioxidant, immunomodulatory, hypolipidemic and anti-inflammatory activities (Gutierrez-Salmean et al., 2015; Bigagli et al., 2017).

Fermentation with sourdough or with beneficial LAB (Lactic Acid Bacteria) strains could further increase the nutritional value of FA-enriched GF products since they provide health benefits to consumers, due to the ingestion of beneficial bacteria and microbial metabolites (Nissen et al., 2020). During fermentation, several VOCs (volatile organic compounds) are synthesized naturally by microorganisms (as secondary metabolites) interacting with the food matrix. VOCs are organic molecules that include esters, alcohols, aldehydes, ketones, phenols, organic acids, terpenes, etc. Beyond their flavoring properties, various reports have shown the potential role of VOCs in human health, including their antioxidant, anti- inflammatory, anti-microbial, and anti-obesity activities (Nissen et al., 2020). Lastly, certain VOCs among the aforementioned compounds have been reported to possess prebiotic activity, e.g. organic acids and terpenes (Nissen et al., 2020a; Nissen et al., 2021). When microalgae are integrated into food, aroma is an important aspect to consider. The presence of sulfuric compounds, diketones, α-ionone, and β-ionone in fresh microalgae biomass is explained by the mechanisms of aroma formation such as enzymatic oxidation of lipids, and enzymatic and chemical degradation of dimethyl sulfoniopropionate (which generates dimethyl sulfide), phenylalanine (generation of benzaldehyde) and carotenoids (generation of ionones) (van Durme et al., 2013). Due to the presence of these unpleasant compounds, the volatilome analysis conducted in this work aimed to predict the aromatic properties of FA-enriched GF products, and to evaluate the impact of the fermentation process on the development of a characteristic flavor profile, in order to improve the aromatic properties of baked goods. Thus, given the common industrial knowledge gap about the quality of protein fortified GF foods, our work aimed to consider the effect of fortification on the volatilome, with respect to VOCs with bioactivity and with respect to prebiotic activity.

CASE STUDY 2: Bread fortified by spirulina

The recipient study is based on a metabolomic approach to evaluate VOC production in a sourdough-fermented GF bread enriched with *A. platensis* and to relate these compounds with the flavoring and health properties of the final product. Besides, processing variables, consumers palatability, and prebiotic activity were pondered and correlations among VOC production were evaluated.

5.1.2. **Materials and methods**

5.1.2.1. Bacterial strains and culture conditions

Microbial strains belonged to the microbial collection of the Department of Agricultural and Food Sciences, University of Bologna (Italy) (Nissen et al., 2020; Nissen et al., 2020b). *Lactobacillus plantarum 98a, Lb. sanfrancisciensis Bb12* and *Saccharomyces cerevisiae LBS* were obtained from 30% (v/v) glycerol stocks stored at −80 °C. Bacteria were propagated in MRS (de Man–Rogosa–Sharpe) broth (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) (dextrose 20%; peptone; 10%; beef extract 8%; sodium acetate 5%; yeast extract 4%; ammonium citrate 2%; dipotassium phosphate 2%; polysorbate 80 1%; magnesium sulfate 0.2%; manganese sulfate 0.05%) at 37 °C for at least 48 h and yeasts in Sabouraud Dextrose broth (Oxoid, Thermo Fisher Scientific, USA) (dextrose 20%; peptone; 10%; pancreatic digest of casein 5%; pancreatic digest of animal tissue 5%; chloramphenicol 0.05gL−1) at 30°C for 24h.

5.1.2.2. Doughs and bread preparation

Flours were commercial organic certified products (**Table S1**). Experimental doughs (approximately 700 g) were prepared according to the formulation reported in **Table S2**. Maize and rice were partially substituted with 5.3% (w/w) FA (Algae Flour) to obtain a GF formulation suitable to be considered as a protein source. The list of samples and their codes are described in **Table 1**. Two types of dough formulations were used: a standard type (ST) including maize and rice flours and an algae type (AT) where FA (5.3% w/w) replaced standard flours. The percentage of FA to be used for enrichment was set to generate a dough with at least 12% of energy derived from a protein that can be claimed the "protein source" and was based on the sensory characteristics of the final products (data not shown), and on a similar approach adopted in the formulation of non-GF fortified bakery products (Niccolai et al., 2019). Both types were used for direct fermentations and for sourdough fermentations. Direct fermentations were as follows: (i) not inoculated (X); (ii) inoculated with Log10 6 CFU mL−1 of an equal LAB mix of *Lb. sanfrancisciensis Bb12* and *Lb. plantarum 98a* (L); (iii) inoculated with Log10 7 CFU mL−1 of *S. cerevisiae LBS* (Y), and were conducted for 18 h at 31 °C. The samples for sourdough fermentations, indicated by a "+" in the labels, were made by replacing 20% of AT and ST dough formulations with 140 g of direct LAB mix 18 h fermented doughs (L) and inoculated with

Log10 7 CFU mL−1 of *S. cerevisiae LBS* (Y). The complete sour- doughs were then fermented for 6 h at 31 °C. As an additional control, a direct fermentation with Y was conducted for 6 h at 31 °C. All fermented doughs were baked at 180 °C for 20 min to produce breads (B). All samples were produced in triplicate in two independent experiments. The doughs were prepared with a kneading machine (Bimby Tm31, Vorwerk, Wuppertal, Germany) setting the program for bread making, then were formed in single steel containers, fermented in a laboratory incubator (MPM Instruments, Srl, Bernareggio, Italy), and baked with an electric oven (Mod.KOABS31X, Electrolux, Stockholm, Sweden).

Table 1. Description of samples codes.

Sample	Description
FA	Flour of Algae (*Arthrospira platensis*)
FM	Flour of Maize
FR	Flour of Rice
AX	Algae dough not inoculated (direct)
AL	Algae dough LAB inoculated (direct)
AY	Algae dough *S. cerevisiae* LBS inoculated (direct)
SX	Standard dough not inoculated (direct)
SL	Standard dough LAB inoculated (direct)
SY	Standard dough *S. cerevisiae* LBS inoculated (direct)
YA+	Algae dough added with sourdough
YS+	Standard dough added with sourdough
AX18	AX fermented 18 h
AL18	AL fermented 18 h
AY18	AY fermented 18 h
SX18	SX fermented 18 h
SL18	SL fermented 18 h
SY18	SY fermented 18 h
YA+6	YA+ fermented 6 h
YS+6	YS+ fermented 6 h
YA6	YA* fermented 6 h
YS6	YS* fermented 6 h
YA+B	Bread from YA+6
YS+B	Bread from YS+6
YAB	Bread from YA6
YSB	Bread from YS6
ALB	Bread from AL18
AYB	Bread from AY18
SLB	Bread from SL18
SYB	Bread from SY18

* same formulations of AY and SY, respectively.

5.1.2.3. Microbial quantification during the process

Microbial quantification was obtained by both culture-dependent and culture-independent protocols. The culture-dependent quantification was done by plating serial dilutions of the samples in sterile physiological solution (NaCl 0.9% w/v). LAB were plated on MRS (de Man–Rogosa–Sharpe) (Oxoid, Thermo Fisher Scientific, USA) agar and cycloheximide (0.1 g L−1) (Sigma, Saint Louis, MO, USA) and incubated aerobically for 48 h at 37 °C. Yeasts were plated on Sabouraud Dextrose Agar (Oxoid, Thermo Fisher Scientific, USA) and chloramphenicol (0.05 g L−1) (Sigma, USA) and incubated aerobically for 24 h at 30 °C. Quantification was calculated as Log10CFU mL−1 (Colony Forming Units per mL). The culture independent protocol was performed by qPCR with the SYBR Green I chemistry, applying genus specific primers (Eurofins Genomics GmbH, Ebersberg, DE) as Lac1 for *Lactobacillus spp.*, then named LAB, (forward: 5′-GCAGCAGTAGGGAATCTTCCA-3′ and reverse: 5′-GCATTYCACCGCTACACATG-3′)17 and ITS 23S for *S. cerevisiae LBS*, then named yeasts, (forward: 5′- GTTTCCGTAGGTGAACCTGC-3′ and reverse: 5′-ATATGCTTAAGTTCAGCGGGT-3′) (Foschino et al., 2004). Extraction of bacterial DNA was obtained with a Nucleo Spin Food DNA Extraction Kit (Macherey Naegel, Duren, Germany) prior to a pre-treatment of 10 min at 20 Hz of ultra-pure water diluted doughs in a sonication bath. Genetic standards were prepared from relative PCR amplicons from pure cultures of the target bacterial species as described previously (Nissen et al., 2020; Nissen et al., 2020b). Templates for qPCR to generate standard curves were amplified by PCR using a ProFlex PCR System apparatus (Thermo Fisher Scientific, USA) with SuperFi Platinum Taq (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Amplicons were purified with a GeneJet PCR Purification kit (Thermo Fisher Scientific, USA). For both the targets, qPCR reactions were performed with a Power-Up Master Mix (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) on a RotorGene 6000 (Qiagen, Hilden, Germany) with the RotorGene Q Series Software 2.3.1 Release (Qiagen, Germany), set and analysed as previously described. (Nissen et al., 2020; Nissen et al., 2021). Sample reactions were conducted in triplicate, with positive, negative, and background controls. Quantification was calculated as GCN per mL (Gene Copy Number per mL) and the value was divided by three (the presumptive copies of a ribosome per cell), expressed as Log10 cells per mL (Nissen et al., 2020; Nissen et al., 2020b).

5.1.2.4. pH changes during the process

The pH was determined at 20 °C with a pH meter (Crison, Alella, Spain) appropriately calibrated with three standard buffer solutions at pH 9.21, pH 4.00, and pH 2.00. The pH values were measured in duplicate at the beginning and the end of fermentation (**Table S3**).

5.1.2.5. Prebiotic score

Stomacher-homogenized (3500 paddle blender, Seward Ltd, Worthing, UK) and sterile physiological solution (NaCl 0.9% w/v) 1:10 (w/v) diluted samples were employed to test for prebiotic potential with the prebiotic score method previously described by Fissore et al. (2015) with modifications (Nissen et al., 2020a). Briefly the method is based on the selective growth of probiotics and enteropathogens on minimal broths enriched with 1% (w/v) homogenized food products in comparison with control sugar (glucose 1% w/v) and prebiotic fructo-oligosaccharides (FOS 1% w/v) from chicory (Sigma, USA). The bacterial type strains *Lb. plantarum 98b, B. bifidum 700795,* and *E. coli 25922* were used, and propagated as previously reported (Nissen et al., 2020a; Fissore et al., 2015). Bacterial loads of the inocula were adjusted with the aid of a spectrophotometer (Tecan M200 Plate Reader, Tecan Trading AG, CH) to obtain a final concentration of 6 Log10 CFU mL−1, afterwards confirmed by culture dependent and independent quantifications. The prebiotic activity score was calculated with the related formula from two independent experiments and the technical triplicate as previously described (Fissore et al., 2015; Huebner et al., 2008), including qPCR quantifications (Nissen et al., 2020a).

5.1.2.6. Solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS)

Evaluation of volatile organic compounds (VOCs) was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV), equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME-GC-MS (solid phase micro-extraction gas chromatography-mass spectrometry) protocol and the identification of volatile compounds were done according to previous reports, with minor modifications (Nissen et al., 2020; Nissen et al., 2021; Nissen et al., 2020b). Briefly, before each head space sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 40 °C. The SPME fiber was exposed to each sample for 40 min and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 0 min, then programmed at 1.5 °C min^{-1} to 65 °C, and finally at 3.5 °C min^{-1} to 220 °C, which was maintained for 20 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were carried out in splitless mode, and helium (3 mL min) was used as the carrier gas. Identification of molecules was carried out by comparing their retention times with those of pure compounds (Sigma, USA) and confirmed by searching mass spectra in the available databases (NIST version 2005 and Wiley version 1996) and the literature

(Nissen et al., 2020; Nissen et al., 2021; Nissen et al., 2020b). Ethyl alcohol, 1,4- butanediol, 2-butanone-3-hydroxy and acetic acid were absolutely quantified in mg kg^{-1} (**Table 3**), while all other VOCs were relatively quantified in percentage.

5.1.2.7. Sensory evaluation

The breads were evaluated after 3 h from baking by 20 semi-trained testers (consumers), that scored the produced breads according to a preference protocol with a scale from 0 (unacceptable) to 7 (excellent) (Huebner et al., 2008). Two independent consumers' tests were performed, and results were marked in a spider chart as average scores for color, aftertaste, smell, taste, crispiness, and overall appreciation of the breads.

5.1.2.8. Statistical analyses

All statistical analyses were performed using TIBCO Statistica 8.0 (Tibco Inc., Palo Alto, CA, USA). Normality was checked with the Shapiro–Wilk test, while homoscedasticity was evaluated with the Levene's test (Plessas et al., 2008). Differences between all samples were evaluated with untargeted Analysis of Variance (ANOVA) set at $P < 0.05$. Multivariate analysis was conducted with principal component analysis (PCA), K-mean clustering, and MANOVA ($P < 0.05$). Pearson correlations were used to generate the heatmap of VOCs prior fermentation. For post hoc test, a Tukey's HSD (honestly significant difference) test was employed ($P < 0.05$). Except for the quantification in mg/Kg of major metabolites, independently normalized data set was proposed for each chemical class of molecules. The data were normalized using the mean centering method. All results are expressed as mean values obtained at least from duplicate batches in two independent experiments (Nissen et al., 2020; Nissen et al., 2021; Nissen et al., 2020b).

5.1.3. **Results**

5.1.3.1. Microbial quantification and pH values during the process

Quantification of LAB and yeasts obtained by plate count and qPCR are shown in **Figure 1**. Results are expressed as Log_{10} cell/g and represent the mean value of culture-dependent and a culture-independent data (Nissen et al., 2020a; Nissen et al., 2021). Accounting yeast top quantification was recorded by direct fermentation with yeast of FA (Algae Flour) added dough after 18 h of fermentation (AY18) (8.62 ± 0.24 Log10 cell/g), that was slightly higher than in the standard dough (SY18). Oppositely, LAB top quantification was scored by standard sourdoughs (YS+6) (11.00 ± 0.14 Log_{10} cell/g) slightly higher than the relative FA added dough (YA+6). In FA added and standard sourdoughs, the microbial load resulted similar either for yeast or LAB quantifications ($P > 0.05$). In

all conditions, direct inoculation of LAB was the starter that mostly reduced pH during fermentation (Table S3). In particular, the algae type (AT) doughs (pH 4.04 ± 0.12) had an acidification milder than their relative standards (ST) ($P > 0.05$). After 24 h of sourdough fermentation, the most acidified dough was still the standard (YS+6), but with no difference to the YA+6 (4.31 ± 0.15).

In all algae types (AT) fermented samples, microbial growth was similar to their standards (ST). This result was confirmed also by the acidification observed in this study, that was similar for AT and ST samples. Acidification is a fundamental parameter for evaluating the effectiveness of fermentation. Dough acidifications seemed slightly but significantly different that may be done by the higher buffering activity of standard doughs partially replaced with FA in AT samples. Among other things, the acid pH reached thanks to the fermentation process contributes to inhibit the development of spoilage and pathogenic microorganisms and to give a pleasant aroma to the bread.

Moreover, FA addition to the dough directly fermented with LAB (AL18), with respect to the standard (SL18), provided a higher production of Ethanol, the most important descriptor for an efficient leavening process. Although the content of 2-Butanone-3-hydroxy, which is essential for the structure and a pleasant aroma of the baked product, decreased in the ALB bread due to the baking loss, it was still higher than that of standard breads.

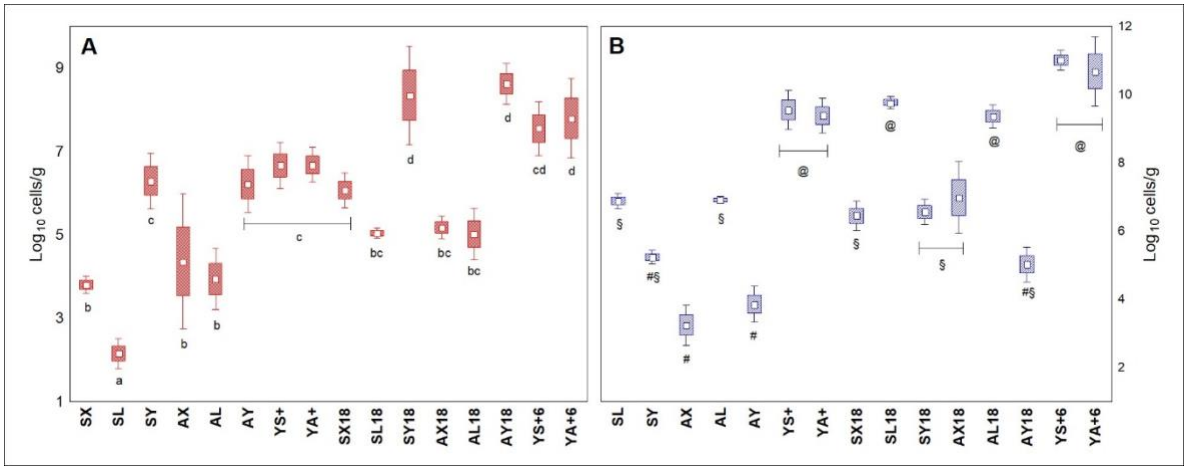

Figure 1. Mean values of absolute quantifications (Log_{10} cells/g) of A) yeasts and B) LAB (Lactic Acid Bacteria) prior baking. Each value is a mean derived from technical duplicates (Log_{10} CFU/mL) and triplicates Log_{10} GCN/mL) from two independent experiments (n = 20). Middle point = mean; box = mean ± standard deviation (SD): whiskers = mean ± SD*1.96. Different letters or symbols indicate statistical significance by Tukey's HSD test (at least $P < 0.05$). For samples abbreviations see **Table 1**.

5.1.3.2. Prebiotic activity

The prebiotic activity was determined according to Fissore et al. (2015), with some modifications, as microbial quantification by qPCR and the use of FOS (Fructo-oligosaccharides from chicory) (Sigma, USA) as prebiotic positive control. Huebner et al. (2008) established a quantitative score to easy describe the extent to which prebiotics foster the selective growth of probiotic species of lactobacilli and bifidobacteria. A given bioactive compound has a positive prebiotic activity score if is rather metabolized by probiotic bacteria and not by opportunistic intestinal ones (Fissore et al., 2015).

The highest score of prebiotic activity towards *L. plantarum* was obtained with the not inoculated algae dough (AX), similar to FOS ($P > 0.05$) (**Table 2**). From this level, there was a reduction in the score of 1.70-fold following the baking step (YA+B) ($P < 0.05$). However, compared to FOS, YA+B did not produce high output ($P > 0.05$).

The same results were observed when the prebiotic activity was assayed versus *B. bifidum*. Indeed, the best outcome was obtained by AX, with no significant differences from FOS ($P > 0.05$). In this case, however, a significant score decrease was observed following baking step ($P < 0.05$), compared to the score recorded for AX.

Again, however, compared to FOS, YA+B did not produce high output ($P > 0.05$). In this work the average of the prebiotic activity versus *L. plantarum* (0.188 ± 0.10) was higher than that versus *B. bifidum* (0.143 ± 0.12), similarly to previous literature (Nissen et al., 2020a).

Anyhow, the two AT samples performed better than the standard samples. Indeed, these latter did not have any prebiotic activity, mainly because were not able to inhibit the growth of enteropathogens.

This result is consistent with previous studies, according to which Spirulina biomass supported the growth of *L. casei, L. acidophilus, Streptococcus thermophilus* and other beneficial bacteria, such as *Bifidobacterium spp.,* while inhibiting the growth of harmful bacteria, such as *Proteus vulgaris, Bacillus subtilis* and *B. pumulis* (Nissen et al., 2019; Bhowmilk et al., 2009; Parada et al., 1998) .This can be attributed to the high content of polysaccharides (PS) or their derivatives, namely oligosaccharides or low-molecular-weight (LMW)-PS which also includes the so-called dietary fibers. In fact, several studies indicate that some of these compounds, also called non-digestible oligosaccharides (NDOs), offer a significant advantage to the host health by stimulating the growth of beneficial bacteria and modulating the composition of the colon microbiota, thus satisfying the criterion of prebiotics (Beheshtipour et al., 2012). For example, alginate oligosaccharide (AlgO) is enzymatically hydrolyzed from alginate and possesses prebiotic properties, which have been shown to stimulate the growth of bifidobacteria, both in in vitro and in vivo (Roberfroid et al., 2000). Agarose oligosaccharides (AO) from agarose enzymatic hydrolysis also exhibit prebiotic effects, stimulating the growth of bifidobacteria and lactobacilli and escaping digestion in the upper gastrointestinal tract

(Han et al., 2019). Part of the strong prebiotic activity score of the AP-enriched samples may be due to the higher level of propionate and butyrate in the AT samples, particularly on the AT breads for butyrate and the AT doughs for propionate (Figure S2A). Indeed, these compounds are known to promote the selective microbial growth of probiotics and beneficial microbes in the intestine (Han et al., 2009), stimulate epithelial immune function (Esgalhado et al., 2017) and modulate the inflammatory response to pathogens (Goverse et al., 2017).

Table 2. Prebiotic score on doughs and breads.

Sample	*Lactobacillus plantarum*	*Bifidobacterium bifidum*
AX	0.344 ± 0.13^{a}	0.342 ± 0.14^{a}
SX	0.122 ± 0.16^{c}	0.077 ± 0.07^{b}
YA+B	0.202 ± 0.12^{b}	0.134 ± 0.08^{b}
YS+B	0.087 ± 0.09^{c}	0.019 ± 0.04^{b}
FOS	0.354 ± 0.15^{a}	0.298 ± 0.11^{a}

Values are means of two independent experiments, two replicates for plate counts and three for qPCR (n = 10). ^{abc}Different letters among a column indicate significative differences by Student's t-test ($P < 0.05$). For samples abbreviations see Table 1.

5.1.3.3. Analysis of volatilome

Volatilome analysis identified more than 250 molecules and relatively quantified approximately 147. For a landscape description of the volatilome, two datasets normalized with the mean centering method were proposed: (i) one including quadruplicates of not fermented cases (n = 36) and 177 molecules (**Figure S1**) and (ii) one including quadruplicates of all experimental cases (n = 116) considering the sums of relative abundances of significant VOCs (ANOVA $P < 0.05$) grouped by chemical classes, employed to compare the not fermented cases to the means of fermented cases and breads cases (**Figure 2**). Afterward, for a more specific investigation and to generate robust data trainings for multivariate analysis, two other options were chosen: (iii) the most abundant VOCs (Ethyl alcohol, Acetic acid, 2-Butanone-3-hydroxy, and 1,4-Butandiol) were set apart and independently quantified in mg/Kg using an internal standard as described previously12,22 (**Table 3**); (iv) all other 93 significant VOCs (ANOVA $P < 0.05$) were super-normalized in five distinct data sets organized by chemical classes (17 organic acids, 21 ketones, 20 aldehydes, 22 alcohols, 13 alkenes) to perform multivariate analyses (PCA, K-Means, and MANOVA) (**Figures 3-7**).

5.1.3.3.1. Quantification of VOCs before fermentation

Considering flours and not fermented doughs, 128 VOCs were detected in ST and 167 in AT. The data set was clustered by Pearson analysis in two major groups, the first including ST not fermented doughs and FM and FR, while the second including ST not fermented doughs and FA (**Figure S1**). A higher quantity and a wider speciation of compounds was identified in AT samples. In most cases, Hexanal and Benzenamine N-ethyl were the two most abundant VOC, reaching the top level in FR and AX samples, but among the entire dataset 8-Heptadecene, (Z) of FA samples was the most abundant. In AT not fermented samples, Heptadecene, 3-Tetradecene, (E), and Butylated hydroxy toluene were the most abundant. Of note, these latter VOC was 1.96-folds higher than in ST samples. As well, Nicotinic acid, propyl ester, Borneol, and Phycocyanin were exclusive signature of AT samples.

5.1.3.3.2. Effect of processing

Either in AT or ST samples, fermentation caused a significant increase of VOCs related to alcohols, aldehydes, and organic acids, while a reduction in alkenes ($P < 0.05$). AT samples after fermentation had the highest load in organic acids, significantly higher than in ST samples. After baking, in AT bread the concentration of total VOCs significantly decreased in comparison to fermented doughs, but VOCs retained in bread were still significantly higher than in not fermented AT samples ($P < 0.05$). AT baked samples in comparison to ST samples had higher abundance of ketones and organic acids, while less abundance of alkanes, amines, and esters ($P < 0.05$) (**Figure 2**).

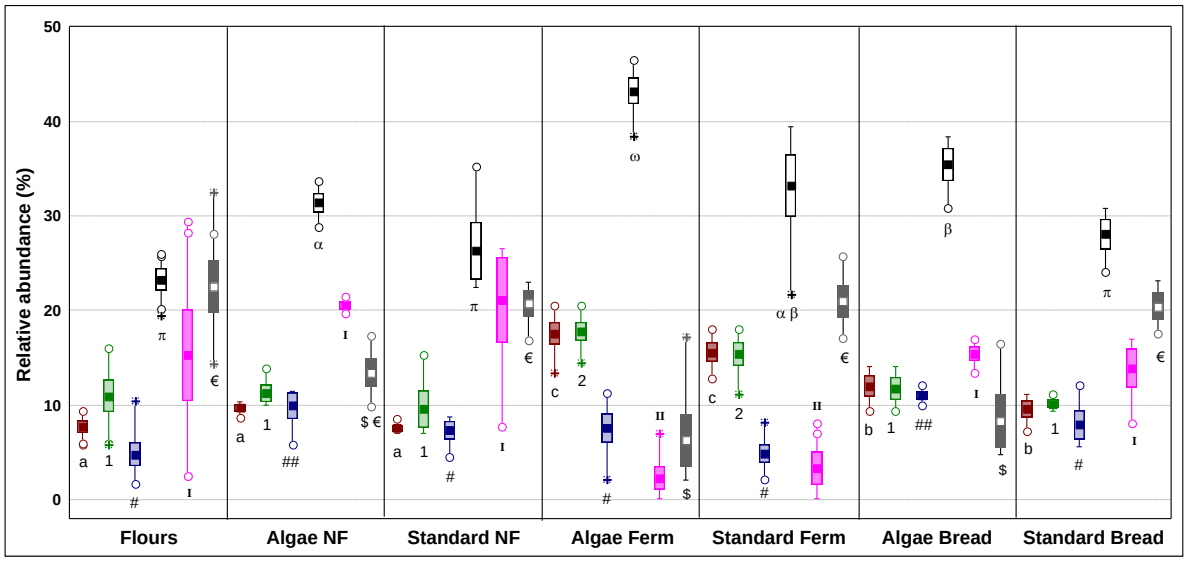

Figure 2. Relative quantification of total volatile organic compounds (VOCs) divided by chemical classes. Different letters indicate different significance values by Tukey's HSD (honestly significant difference) test ($p < 0.05$). Sample abbreviations: NF = not fermented; Ferm = fermented. Box = mean value; Rectangles = mean ± Standard Deviation (SD); Whiskers = min and max; circles = outliers; Asterisks = extremes. Red plots = Alcohols; Green plots = aldehydes; Blue plots = Ketones; Black and White plots = Organic acids; Fuchsia plots = Alkenes; Gray plot = others (amines, alkanes, and esters).

5.1.3.3.3. Quantifications of the main fermentation metabolites

Quantification of main fermentation metabolites (mg/Kg of fermented matrix) is reported in **Table 3.** Ethanol produced in yeast fermented algae dough (AY18) did not show a significantly different score from its control (SY18) ($p > 0.05$) (**Table 3**), and the same trend was observed when the fermentation process was performed by sourdough (YA6 and YS6) ($p > 0.05$). 1,4-Butanediol in FA-enriched doughs scored the greatly highest concentration when directly fermented with yeast for 18 h (AY18) while FA-enriched sourdoughs (YA+6) produced 1.24-folds more 1,4-Butanediol than the control (YS+6) ($p > 0.05$). In baked samples, the sourdough fermented YA+ (YA+B) had 5.21-folds more 1,4-Butanediol than the control (YS+B) ($p < 0.05$). In comparison to the control, FA-enriched doughs directly fermented by LAB (AL18) produced 1.25-fold more 2-Butanone-3-hydroxy than the control (SL18) ($P < 0.05$), but in standard bread (SLB) this compound was retained 1.58-folds more ($p > 0.05$) than in FA-enriched bread (ALB). YS+6 produced 1.14-folds more Acetic acid than the FA-enriched dough (YA+6) ($p > 0.05$), and the same result was observed in baked samples. In fact, YS+B produced 1.69-folds more Acetic acid than YA+B ($p < 0.05$).

Table 3. Quantification (mg/Kg) of major fermentation compounds by SPME GC-MS with a close relative internal standard compound.

Sample	Ethyl alcohol	1,4-Butanediol	2-Butanone-3-hydroxy	Acetic acid
FR	n.d.	n.d.	n.d.	n.d.
FM	n.d.	n.d.	n.d.	n.d.
FA	n.d.	n.d.	n.d.	n.d.
AX	n.d.	n.d.	n.d.	0.20 ± 0.04^{a}
AL	n.d.	n.d.	n.d.	0.28 ± 0.09^{a}
AY	n.d.	n.d.	n.d.	n.d.
SX	tr.	n.d.	n.d.	n.d.
SL	0.23 ± 0.02^{a}	n.d.	n.d.	n.d.
SY	tr.	n.d.	n.d.	n.d.
YA+	5.55 ± 0.87^{c}	6.69 ± 1.16^{d}	0.51 ± 0.16^{a}	0.69 ± 0.42^{a}
YS+	4.03 ± 0.72^{c}	6.43 ± 1.32^{d}	0.37 ± 0.09^{a}	0.33 ± 0.11^{a}
AX18	14.69 ± 2.65	17.11 ± 2.12^{e}	8.44 ± 1.13^{bc}	2.95 ± 0.45^{bc}

AL18	22.57 ± 4.76^{e}	19.73 ± 0.97^{e}	11.09 ± 1.99^{d}	8.30 ± 0.99^{d}
AY18	26.45 ± 3.97^{e}	23.37 ± 3.01^{e}	13.88 ± 2.08^{d}	6.25 ± 1.56^{c}
YA+6	22.77 ± 2.78^{d}	26.56 ± 2.32^{e}	9.13 ± 1.09^{c}	7.46 ± 1.33^{cd}
YA6	16.53 ± 1.95^{d}	17.88 ± 1.45^{e}	8.02 ± 1.45^{bc}	5.99 ± 1.11^{c}
SX18	9.61 ± 2.69^{cd}	7.77 ± 0.34^{d}	7.11 ± 2.02^{bc}	3.46 ± 0.87^{bc}
SL18	19.12 ± 2.88^{d}	14.69 ± 1.49^{de}	8.84 ± 0.99^{c}	9.15 ± 1.57^{d}
SY18	28.02 ± 4.09^{e}	26.01 ± 1.17^{e}	9.08 ± 0.85^{c}	8.64 ± 1.69^{d}
YS+6	19.86 ± 3.78^{d}	28.75 ± 2.21^{e}	7.11 ± 2.02^{bc}	8.54 ± 2.41^{d}
YS6	15.77 ± 2.78^{d}	22.11 ± 2.03^{e}	6.12 ± 0.55^{b}	6.76 ± 0.55^{c}
ALB	0.71 ± 0.08^{b}	0.24 ± 0.02^{a}	6.78 ± 0.89^{b}	1.78 ± 0.65^{b}
AYB	0.54 ± 0.11^{ab}	0.34 ± 0.06^{a}	7.53 ± 1.96^{bc}	0.44 ± 0.09^{a}
SLB	0.83 ± 0.06^{b}	0.31 ± 0.01^{a}	4.45 ± 0.44^{b}	1.99 ± 0.78^{b}
SYB	0.45 ± 0.09^{ab}	1.02 ± 0.17^{b}	6.77 ± 0.99^{b}	tr.
YAB	0.55 ± 0.06^{ab}	1.76 ± 0.35^{b}	6.00 ± 0.78^{b}	0.30 ± 0.06^{a}
YSB	tr.	1.45 ± 0.78^{b}	5.45 ± 1.30^{b}	tr.
YA+B	0.69 ± 0.08^{ab}	0.47 ± 0.09^{a}	7.54 ± 1.76^{bc}	2.49 ± 0.21^{b}
YS+B	1.47 ± 0.34^{b}	2.45 ± 0.43^{c}	6.89 ± 1.45^{bc}	4.22 ± 1.04^{c}

Values are means of two replicates and two different batches (n = 4). ^{abc}Different letters among a column indicate significative differences by Tukey's HSD test (P < 0.05). n.d. = not detected (< 0.1 mg/Kg); tr. = traces (0.1 – 0.2 mg/Kg). For samples abbreviations see Table 1.

5.1.3.3.4. Multivariate analysis of VOCs organized by different chemical classes

Organic acid

From analysis of variance including all samples (n = 58), 16 organic acids resulted significantly different ($p < 0.05$) and on PCA their loadings on independent variables (**Figure 3A**) were clustered in four sets by K-means analysis (**Figure S2A**). AT samples were grouped in two clusters: in Cluster 1 collecting the fermented doughs and in Cluster 4 the breads. Cluster 1 was mainly described by six significant ($p < 0.05$) VOCs, as: Propanoic acid hydroxy, Nicotinic acid, Ethyl acetate, Propanoic acid, Nonanoic acid, and Thiophene acetic acid. By K-means clustering analysis (**Figure S2A**), these six VOCs accounted to be produced for averagely the 50% of total cases by the members of this cluster. In particular, Cluster 1 was addressed responsible of around 66% of Propanoic acid and 56% of Nonanoic acid productions.

Cluster 4 consisted of AT breads and was described by seven significant ($p < 0.05$) organic acids, namely: Dehydroacetic acid, Butanoic acid, Pentanoic acid, Hexanoic acid, Heptanoic acid, Octanoic acid, and n-Hexadecanoic acid. In particular, the contribution of AT breads on the production of these VOCs among all cases was pretty large, comprised from the 89% of Butanoic acid to the 66% of Octanoic acid. Lastly, the abundances of Heptanoic acid and Octanoic acid found in this cluster were the two top of the dataset of organic acid (**Figure S2A**). With MANOVA ($p < 0.05$) the contribution of specific cases from selected categories (matrix, starter, time of fermentation, and process stage) on

the production of these VOCs was evaluated. Considering the matrix (**Figure 3b**), Heptanoic acids was found to be a rich and exclusive signature of AT samples, as well as minor Nicotinic acid, while Propanoic acid hydroxy was almost 2-folds higher in AT samples. Considering the starters (**Figure 3c**), Butanoic and Hexanoic acids were mainly produced by LAB, while a higher proportion of Propanoic acid hydroxy was made by sourdoughs. This latter VOC was quadrupedally produced after 24 h of fermentation (**Figure 3d**) but was subjected to a baking loss by almost 8-folds (**Figure 3e**). After the baking stage, an increase in the production of n-Decanoic acid was observed (**Figure 3e**), which is a MCFA (medium-chain fatty acid) with bioactive characteristic.

In our study, the organic acids profile of the AT samples was superior to the standard ones. Principally, a higher speciation and greater abundance of organic acids was found after fermentation of AT samples (e.g. Lactic acid and Propanoic acid) and was kept up to the final product (e.g. Butanoic acid and MCFAs). According to the previous study (Granato et al., 2014; Correa et al., 2017), the increased concentration of short-chain or medium-chain organic acids depended mainly on the fermentation process. Propanoic and Lactic acid are flavoring compounds, determining typical sharp, acid, vinegar taste, with a buttery nuance given by Lactic acid (Joya et al., 2020), but are also involved in the quality and safety of fermented foods due to their antimicrobial activity in baked goods, inhibiting ubiquitous bacilli, deteriorating microbes and food-borne pathogens (Ross et al., 1996). Butanoic acid production in our study was mainly derived by LAB fermentation, as reported by several authors (Petel et al., 2017; Bartkiene et al., 2019; Hati et al., 2019). This compound, along with Propanoic acid, fits the new definition of prebiotics (Fu et al., 2019), according to which a prebiotic is a compound that selectively stimulates the growth and/or activity of gut bacteria associated with health and to well-being, thus excluding opportunists or pathogens. In particular, Butanoic acid is known to form the main energy source for intestinal epithelial cells and affects a wide range of cellular functions that affect colon health (Moens et al., 2019), while Propanoic acid is known to promote the growth of probiotic commensals of bifidobacterial (Gibson et al., 2017). Hexanoic acid is a volatile compound resulting from the fermentation carried out by lactobacilli or yeast and responsible for the inhibition of molds in bread (Joya et al., 2020). However, our results show an increase in Hexanoic acid following the baking phase and this may have been caused by the splitting of hydroperoxides (Goverse et al., 2017) generated by lipoxygenases during the fermentation phase. Hexanoic acid and Nonanoic acid are medium-chain fatty acids (MCFA) known for their effectiveness in the excessive consumption of calories, inducing weight loss (Rios-Covian et al., 2016). MCFAs, in fact, are considered health-related compounds as they protect against insulin resistance during calorie excess (Guinet et al., 1994). However, Hexanoic acid and Nonanoic acid have a cheese, waxy, fatty, goat scent that is sensorially unpleasant and the modulation of their content

should be expected for commercial development. We have to consider that the high amount detected in the breads could have impacted the low score of the sensory test (**Figure S3**).

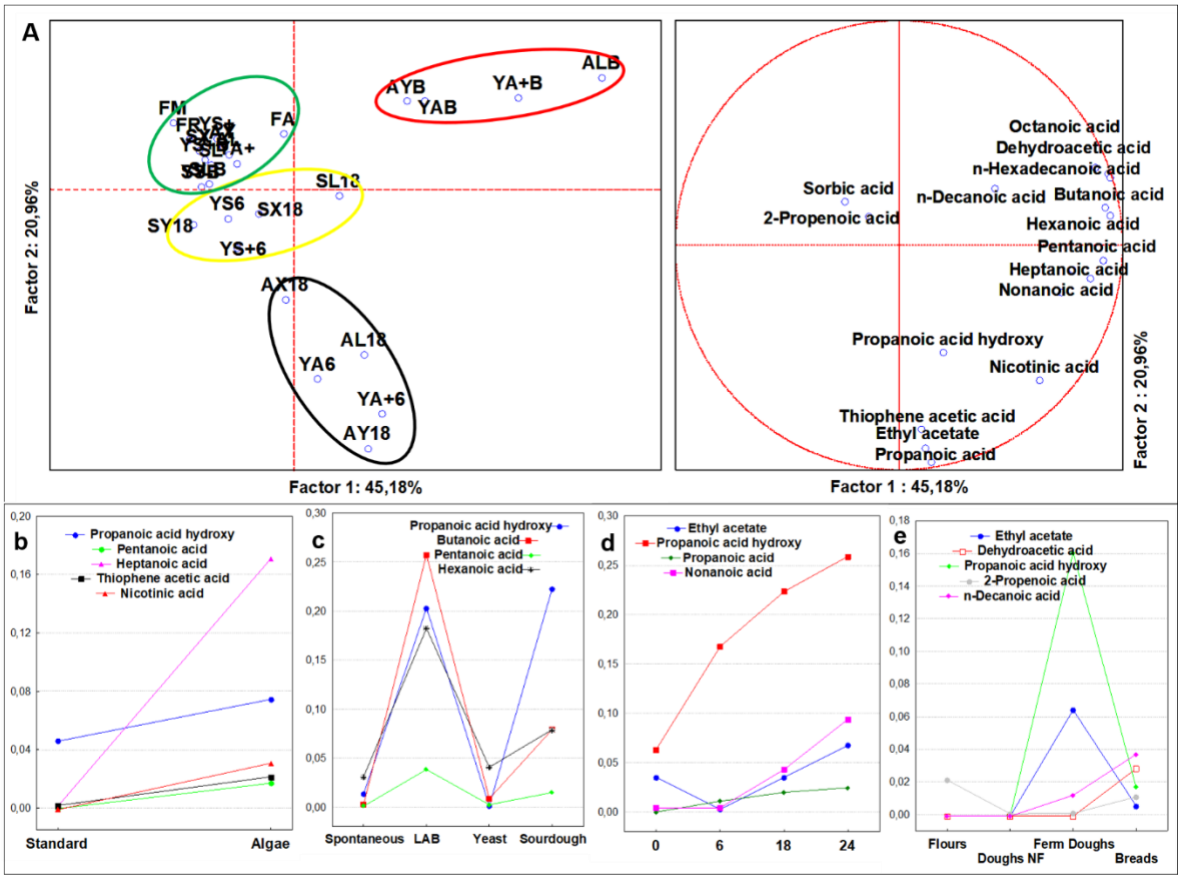

Figure 3. (A) Principle component analysis (PCA) of cases and variables on organic acids (ANOVA $p < 0.05$); (b) MANOVA categorized for the matrix (at least $p < 0.05$); (c) MANOVA categorized for the starters (at least $p < 0.05$), LAB = lactic acid bacteria; (d) MANOVA categorized for the time of fermentation (at least $p < 0.05$), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (e) MANOVA categorized for the process stages (at least $p < 0.05$); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Ketones

From analysis of variance including all samples (n = 58), 19 ketones resulted significantly different ($p < 0.05$) and on PCA their loadings on independent variables (**Figure 4A**) were clustered in three sets by K-means analysis (**Figure S2B**). AT samples were grouped in two clusters: in Cluster 2 the breads and the algae flour and in Cluster 3 the doughs. Cluster 2 was mainly described by six significant ($p < 0.05$) VOCs, as: 2-Pentanone, 2-Heptanone, 7-Octen-2-one, 2,7-Octadienone, 3-Nonen-2-one and 2,(3H)-Furanone,5-ethyldihydro. By K-means clustering analysis (**Figure S2B**), these six VOCs accounted to be produced for averagely the 90% of total cases by the members of this cluster. In particular, Cluster 2 was addressed responsible of around 95% of 3-Nonen-2-one and 100%

of 7-Octen-2-one productions. Cluster 3 consisted of AT doughs and was described by three significant ($p < 0.05$) ketones, namely: Benzofuranone, Mint furanone and Nepetalactone. In particular, the contribution of AT doughs on the production of these VOCs among all cases was quite variable, ranging from 45% of Benzofuranone and Mintfuranone to 90% of Nepetalactone.

With MANOVA ($P < 0.05$) the contribution of specific cases from selected categories (matrix, starter, time of fermentation, and process stage) on the production of these VOCs was evaluated. The matrix (**Figure 4b**) confirmed what was highlighted by the K-means, Nepetalactone was associated to AT samples. Considering the starters (**Figure 4c**), Spontaneous fermentation produced 2-Undecanone 25-folder higher than LAB and sourdough, while Nepetalactone was produced mainly by yeasts after fermentation, however, decreased after cooking (**Figure 4e**). In contrast to Nepetalactone, 2-Pentanone and 2-Decanone increased after cooking.

AT samples were described by a larger speciation and abundance than ST samples. In particular, within the AT samples, the greatest abundance was found in AT breads, probably indicating the incidence of baking step in ketones production. In AT breads were founded ketones with a pleasure aroma, such as 2-Pentanone and 2-Heptanone. 2-Pentanone is described as sweet, fruity aroma and is found both in sourdough and yeast bread (Petel et al., 2017). On the other hand, 2-Heptanone is found in wheat or rye sourdough and confers a typical aroma described as fruity, spicy, sweet, coconut (Rego Costa et al., 2012). Contrariwise, these two aromatic VOCs were found only to a much lesser extent in ST samples. AT doughs were almost exclusively responsible for the Nepetalactone production which, however, was lost, even if not totally, during the baking step. Nepetalactone has been found in the essential oils of several Nepeta species (*Lamiaceae/Labiatae*), which bacteriostatic, fungistatic and antiviral activities have been attributed to nepetalactones (Lundsgaard et al., 2020).

Aldehydes

From analysis of variance including all samples (n = 58), 20 aldehydes resulted significantly different ($p < 0.05$) and on PCA their loadings on independent variables (**Figure 5A**) were clustered in four sets by K-means analysis (**Figure S2C**). AT samples were grouped in two specific clusters: in Cluster 3 the breads and in Cluster 4 the not fermented doughs. On the other hand, AT fermented doughs were grouped in Cluster 1 together with ST doughs. Cluster 3 (AT breads) was positioned in quadrant II and was mainly described by five significant ($p < 0.05$) VOCs, as: Pentanal, 2-Hexenal (E), 2,4-Heptadienal (E,E), Dodecanal and 9-Octadecenal, for averagely the 80% of total cases. 2,4-Heptadienal, (E,E), a derivative of sorbic acid, has pleasant, green, floral flavor (Joya et al., 2020) and is found in camelina oil, raw adzuki beans45, and perilla seeds oil46 with an antifungal efficacy (Hussein et al., 2021). In particular, around 95% of Heptadienal (E,E) and 90% of Hexenal (E)

productions (K-means clustering, Figure S2C). Cluster 4 consisted of AT not fermented doughs, and was described by three significant ($p < 0.05$) aldehydes, namely: 2-Butenal,2-methyl (E), Benzaldehyde and Hexanal, for the 85%, 70%, and 85% of total cases (K-means clustering, Figure S2A). With MANOVA ($p < 0.05$) the contribution of specific cases from selected categories (matrix, starter, time of fermentation, and process stage) on the production of these VOCs was evaluated. Considering the matrix (**Figure 5b**), Furfural was found to be an exclusive signature of ST samples, as opposed to 2,4-Heptadienal (E,E), 2,6-Nonadienal (E,E), 2,4-Decadienal and Dodecanal, which were characteristic of AT samples. Considering the starters (**Figure 5c**), Furfural was mainly associated to LAB, while a higher proportion of 2,4-Decadienal and 2,6-Nonadienal (E,E) was made by Yeast. Sourdough produced an amount of 2-Hexenal (E) comparable to that produced by Yeast. While the production of Hexanal, Nonanal and Benzaldehyde decreased from 0 to 6 h of fermentation, the production of Acetaldehyde showed an opposite trend, increased approximately 20-folds from after 6 h of fermentation (**Figure 5d**). Indeed, this compound is derived, besides by Maillard reaction, by lipid oxidation and yeast fermentation and was described to have a pungent, ethereal, fruity, floreal, green, roasted, malty odor (Joya et al., 2020). It was found in fermented sourdough and in breads directly fermented with yeast (Joya et al., 2020). Fermented doughs, both AT and ST samples, were almost exclusively characterized by Acetaldehyde production.

Hexanal was an exclusive signature of NF doughs, while Furfural, Heptadienal, (E,E), and Butanal methyl were characteristic of baking stage (**Figure 5e**). Furfural, that has a leading role in the aroma of bakery products (Joya et al., 2020), was absent in the AT samples but was present only in the ST samples. This result represents an additional value because Furfural was recently investigated as a potential carcinogen (Bi et al., 2021; Lee et al., 2020).

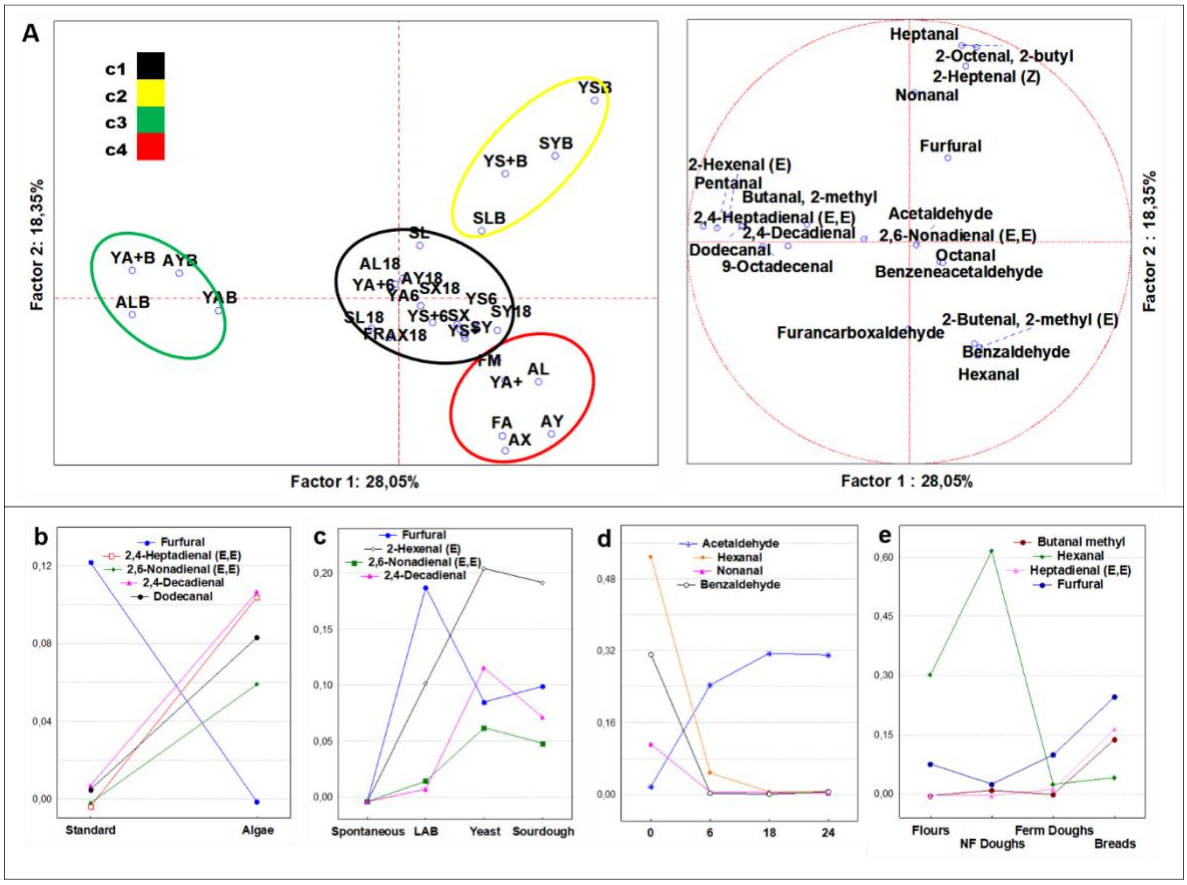

Figure 5. (A) Principle component analysis (PCA) of cases and variables on aldehydes (ANOVA $p < 0.05$); (b) MANOVA categorized for the matrix (at least $p < 0.05$); (c) MANOVA categorized for the starters (at least $p < 0.05$), LAB = lactic acid bacteria; (d) MANOVA categorized for the time of fermentation (at least $p < 0.05$), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (e) MANOVA categorized for the process stages (at least $p < 0.05$); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Alcohols

From analysis of variance including all samples (n = 58), 22 alcohols resulted significant ($p < 0.05$) and on PCA their loadings on independent variables (**Figure 6A**) were clustered in four sets by K-means analysis (**Figure S2A**). AT samples were grouped in three clusters: in Cluster 1 the fermented doughs, in Cluster 3 the not fermented doughs and in Cluster 4 the breads. Cluster 1 was mainly described by five significant ($P < 0.05$) VOCs, as: 1-Pentanol, 3-Cyclohexen-1-ol, 3-methyl, 1-Heptanol, 2,4-Decadien-1-ol, (E,E) and Borneol. By K-means clustering analysis (**Figure S2D**), Cluster 1 (AT fermented doughs) was responsible for the 96% of 1-Pentanol and of 2,4-Decadien-1-ol, (E,E) productions. as well as the 92% of 1-Heptanol and of Borneol productions. 1-Heptanol was reported to be present in rice, soybean, rye and wheat flours or products, and is used as a flavoring agent conferring a typical olfactory issue described as musty, pungent, leafy, green (Ma et al., 2021). 1-Heptanol instead is still associated to sourdough, but from our results was not retained in breads

(Joya et al., 2020). Borneol is considered a bioactive molecule and is reported to modulate beneficially the gut microbiome (Srivastava et al., 2018; Qi et al., 2019: Chen et al., 2013) and to possess anti-inflammatory and antioxidant activity (Cusimano et al., 2020). The exclusive presence in AT products can be considered an added value. However, only a small amount was retained after the cooking phase (about 10% retained). Cluster 3 (AT not fermented doughs) was characterized by lower speciation made by just 11 alcohols and by a typical signature made by Phenylethyl alcohol. Lastly, Cluster 4 (AT breads) was the exclusive producer of Mequinol and Thymol. Thymol is a monoterpene phenol that has the same bioactive characteristics as borneol. The presence of this alcohol in the finished product can have a positive effect on conservation thanks to its ability to inhibit spoilage microbes (Diniz-Silva et al., 2020). In particular, this volatile compound has strong antifungal activity including *Aspergillus spp.* e *Penicillium spp.* (Korona-Glowniak et al., 2020). With MANOVA ($p < 0.05$) on the matrix (**Figure 6b**), 2-Octen-1-ol, (E) was found to be an exclusive signature of AT samples, while Phenylethyl alcohol was almost 2-folds higher in ST samples. Considering the starter (Figure 6c), 2-Octen-1-ol, (E) and 2,4-Decadien-1-ol, (E,E) were mainly produced in the presence of yeasts and after 24 hours of fermentation, while 2-Furanmethanol and Phenylethyl decreased after 6 h of fermentation (**Figure 6d**), while 2-Decanol appeared to be a characteristic signature of baking stage (**Figure 6e**).

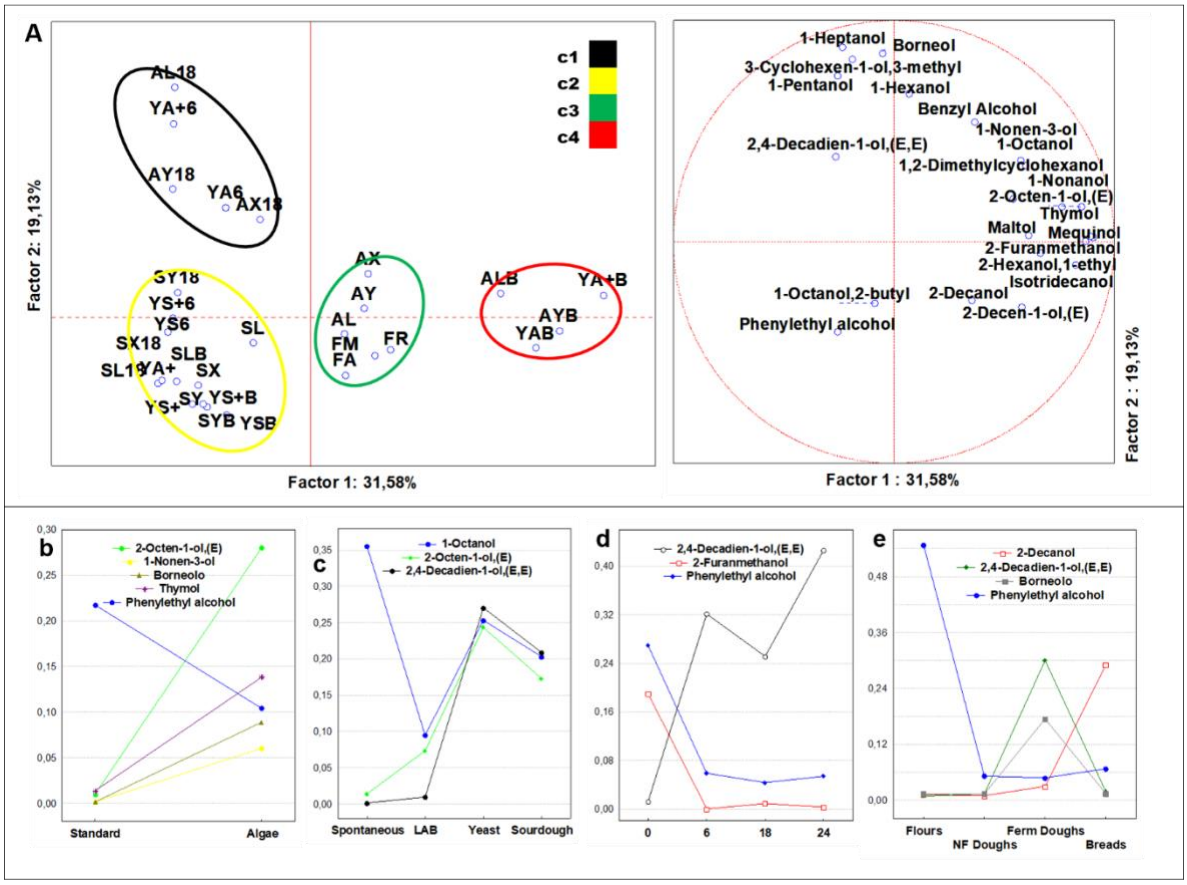

Figure 6. (A) Principle component analysis (PCA) of cases and variables on alcohols (ANOVA $p < 0.05$); (b) MANOVA categorized for the matrix (at least $p < 0.05$); (c) MANOVA categorized for the starters (at least $p < 0.05$), LAB = lactic acid bacteria; (d) MANOVA categorized for the time of fermentation (at least $p < 0.05$), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (e) MANOVA categorized for the process stages (at least $p < 0.05$); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

Alkenes

From analysis of variance including all samples (n=58), 13 alkenes resulted significantly different ($p < 0.05$) and on PCA their loadings on independent variables (**Figure 7A**) were clustered in four sets by K-means analysis (**Figure S2E**). AT samples were grouped in two clusters: in Cluster 1 the breads and in Cluster 4 the fermented and not fermented doughs. Cluster 1, consisted of AT breads, was mainly described by five significant ($p < 0.05$) VOCs, as: Azulene, 1-Heptadecene, (Z), 5-Octadecene, (E), Naphthalene and Phycocyanin. Phycocyanin, a blue photosynthetic pigment widely used in foods and cosmetics (Bansod et al., 2020), occurs naturally in the cyanobacterium *A. platensis* (Guynot et al., 2003) and *Geitlerinema spp.* (Ribes et al., 2017), the eukaryotic algae *Rhodophytesand Cryptophytes* (Mohammadi-Gouraji et al., 2019). This photosynthetic pigment has a peptide nature and has a low thermal stability (Kumar et al., 2014; Patel et al., 2018), in fact our results show a high quantity of it associated with AT doughs, while only a small part is associated to AT breads.

CASE STUDY 2: Bread fortified by spirulina

Phycocyanin is often used as nutritional supplement and has great potential benefits for human nutrition and health, as it contains all the essential amino acids (Lauceri et al., 2018). Phycocyanin has significant anti-oxidative, anti-inflammatory, hepatoprotective, and radical scavenging properties (Patel et al., 2018).

By K-means clustering analysis (**Figure S2E**), these five VOCs accounted to be produced for averagely the 55% of total cases by the members of this cluster. In particular, Cluster 1 (AT breads) was addressed responsible of around 85% of Azulene and 92% of 5-Octadecene, (E) productions. Azulene is a blue organic chromophore, found in nature, having two aromatic rings. It is an isomer of naphthalene and has a similar odor, but the color of the crystal is dark blue (Chaiklahan et al., 2012).

Cluster 4 (AT doughs) was described by almost any aldehydes, and it was addressed by higher concentrations of 7-Hexadecene, (Z) and Phycocyanin, in particular of 90% and 95%, respectively (**Figure S2E**). With MANOVA ($P < 0.05$) on matrix (Figure 7b), Azulene, 1-Heptadecene, (Z), 5-Octadecene, (E) and Phycocyanin were found to be a particular characteristic of AT samples, while 3-Undecene,6-methyl, (E) was characteristic of ST samples. This latter VOC was mainly produced by yeast, while a higher proportion of Napthalene was made by sourdough. LAB, on the other hand, were the main producers of Thiophene, 2-decyl (**Figure 7c**). Considering the time of fermentation (**Figure 7d**), Phycocyanin was highly delivered after 24 h of fermentation but it was not retained after baking (**Figure 7e**). 4-Undecene, 3-methyl, (Z) was a compound characteristic of NF doughs, which decreased with fermentation and baking stages (**Figure 7e**).

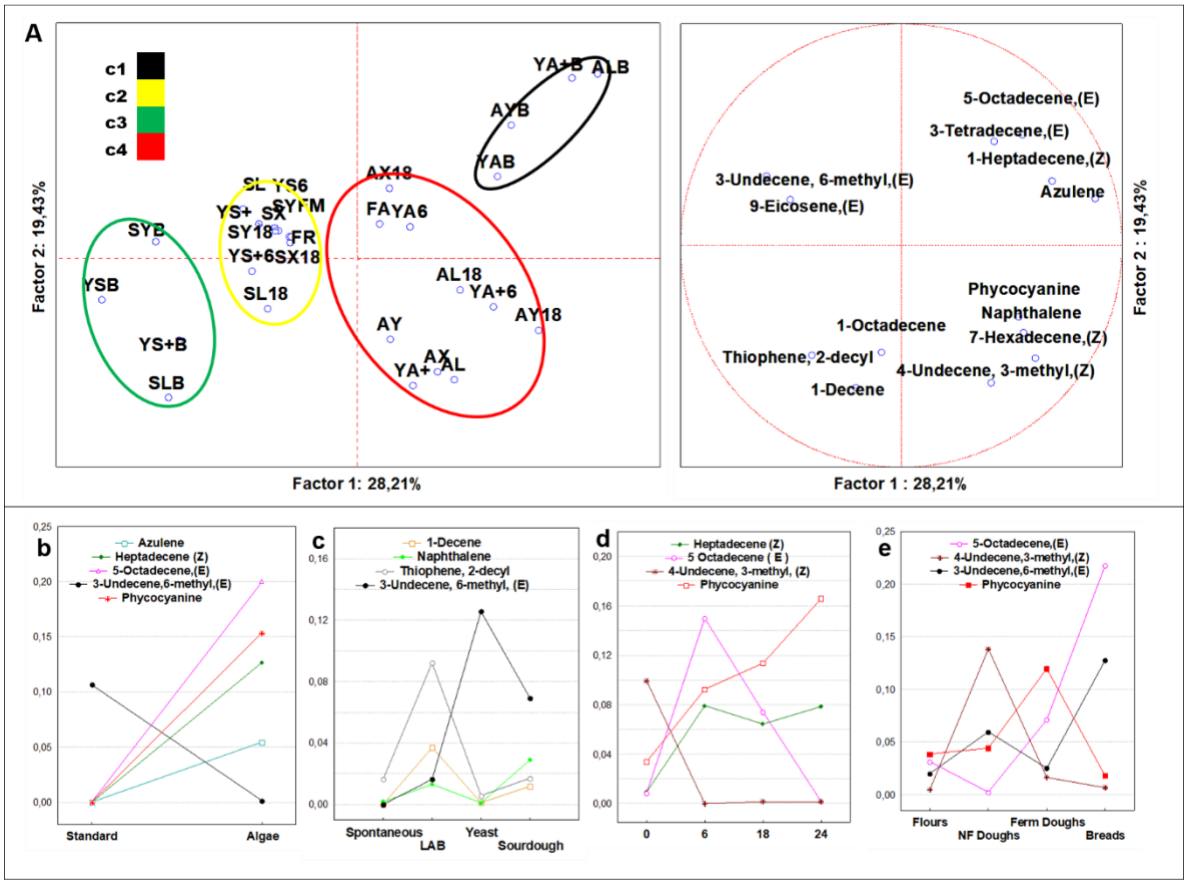

Figure 7. (A) Principle component analysis (PCA) of cases and variables on alkenes (ANOVA $p < 0.05$); (b) MANOVA categorized for the matrix (at least $p < 0.05$); (c) MANOVA categorized for the starters (at least $p < 0.05$), LAB = lactic acid bacteria; (d) MANOVA categorized for the time of fermentation (at least $p < 0.05$), 0 = 0 h, 6 = 6 h at 31 °C, 18 = 18 h at 31 °C, 24 =18 h + 6 h at 31 °C; (e) MANOVA categorized for the process stages (at least $p < 0.05$); NF = not fermented; Ferm = fermented. For samples abbreviations see Table 1.

5.1.4. **Conclusions**

In this work, FA was used to formulate a GF bread enriched in proteins. The bread was quantitatively and qualitatively analyzed throughout the process to evaluate fermentation performances and volatilome composition. The metabolomic profiles of FA-enriched GF breads were considered to investigate the potential of AP as a vehicle for the addition of flavoring and bioactive compounds in bakery products. Multivariate analysis on VOCs provided a deeper description of the effects of AP addition and sourdough fermentation process on flavoring and bioactive compounds, mainly evidencing an increased concentration of antimicrobial compounds, a larger spectrum of bioactive VOCs, and a typical flavoring profile. The addition of FA and the use of different fermentation types gave rise to specific VOCs profile predicting the organoleptic characteristics of bread. AT breads were characterized by green floral nuances derived from the aldehyde content, as well by musty and pungent traits ascribed by that of alcohols, or by sweet and fruity recall given by the ketones profile.

In contrast, the higher presence of hexanoic and nonanoic acids could have contributed to the unpleasant sensorial evaluation scored in this study. Considering the bioactivity of the compounds found in AT samples, the presence of Thymol and Borneol, as well as that of Phycocyanin or SCFAs and MCFAs, as well as Nicotinic acids, represent an important nutritional and functional added value. Even if the content of some of these compounds should be controlled during the process, as some of them are not retained in baked breads, e.g. Phycocyanin and Nicotinic acid. The enrichment with AP could be indicated even as a solution to reduce harmful furfural in the final product.

It is important to mention that the health potential of bioactive compounds delivered in the experimental breads is hardly predictable, because of their high baking loss and consequently the estimation of their bioactivity in humans is hardly predictable. Moreover further studies coupling volatilome analysis to sensorial assessment are needed to understand how the food processing may influence consumers' acceptance. Finally, the evaluation of the shift of VOCs could represent a comprehensive, sensitive, and reliable method guiding the formulation of innovative food with enhanced nutritional value.

5.1.5. **References**

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5.1.6. **Supplementary materials**

Supplementary Table S1. Proximate flours contents.

Flour types	Protein (%)	Carbohydrates (g)	Fiber (g)	Fats (g)
Rice[1]	7.0	79.0	1.0	0.5
Maize[2]	8.5	76.1	2.1	1.3
Algae[3]	31.0	3.1	49.0	8.1

Commercial organic flours from: [1]rice (Molino Rossetto SpA, Pontelongo, Italy), [2]maize (Molino Rossetto SpA, Italy), [3]algae-Spirulina (Erbavoglio Production Srl, San Zeno Naviglio, Brescia).

Supplementary Table S2. Dough formulations.

Dough types	Rice[1] (g)	Maize[2] (g)	Algae[3] (g)	Sourdough (g)	NaCl (g)	sterile water (ml)	HPMC[4] (g)
A	308.61	75.20	15.87		4.00	340.00	5.60
YA+	246.88	60.16	12.70	140.00[5]	3.20	272.00	4.48
S	309.40	90.60			4.00	340.00	5.60

YS+	255.52	64.77	140.00[6]	3.20	272.00	4.48

Commercial organic flours from: 1rice (Molino Rossetto SpA, Pontelongo, Italy) 2maize (Molino Rossetto SpA, Italy), 3algae – Spirulina (Erbavoglio Production Srl, San Zeno Naviglio, Brescia); 4HPMC = Hydroxy propyl methyl cellulose (Bioline Integratori, Canaro, Italy); 5LAB fermented algae enriched dough; 6LAB fermented maize/rice dough

Supplementary table S3. pH values

Sample	**Baseline**	**End of fermentation**
SX	6.24 ± 0.04[c]	4.70 ± 0.08[b]
SL	5.35 ± 0.04[b]	3.60 ± 0.08[a]
SY	5.95 ± 0.03[c]	4.92 ± 0.15[b]
AX	6.27 ± 0.05[c]	5.35 ± 0.17[b]
AL	5.22 ± 0.04[c]	4.04 ± 0.12[a]
AY	6.16 ± 0.03[c]	4.98 ± 0.21[b]
YS	5.80 ± 0.03[c]	5.92 ± 0.06[c]
YS+	5.29 ± 0.09[b]	4.22 ± 0.09[a]
YA	6.11 ± 0.07[c]	5.05 ± 0.11[c]
YA+	5.08 ± 0.12[b]	4.31 ± 0.15[a]

CASE STUDY 2: Bread fortified by spirulina

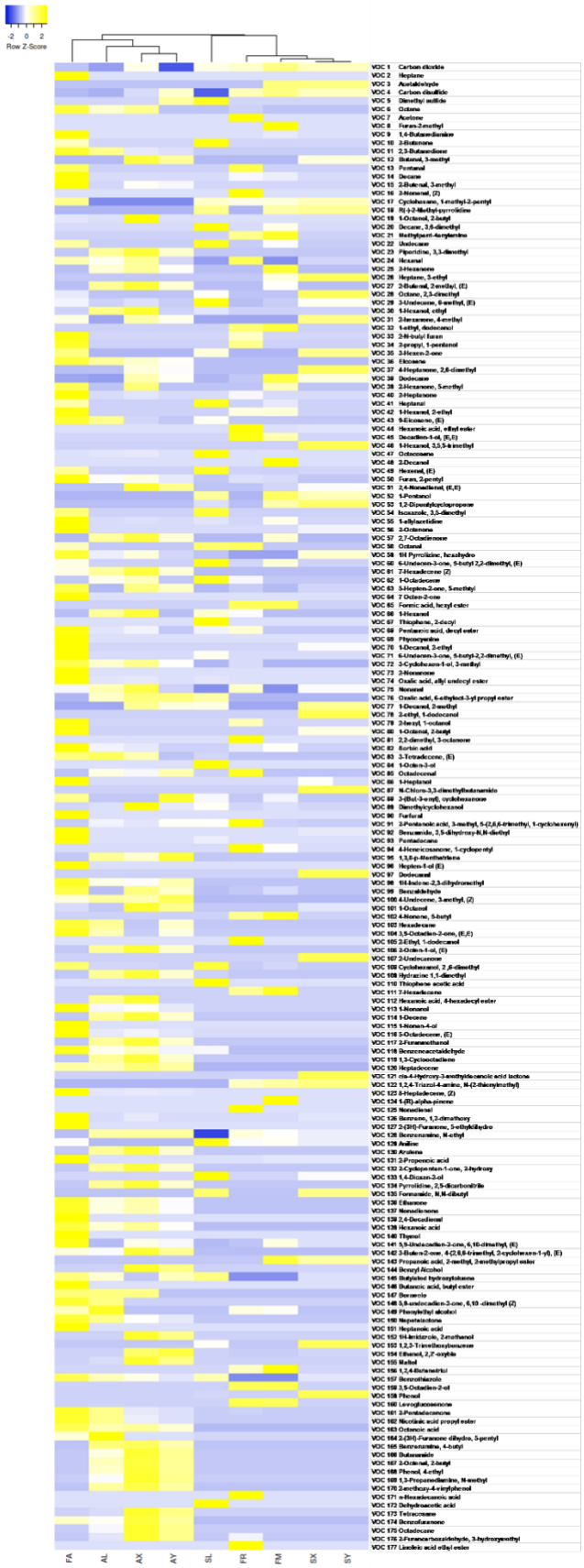
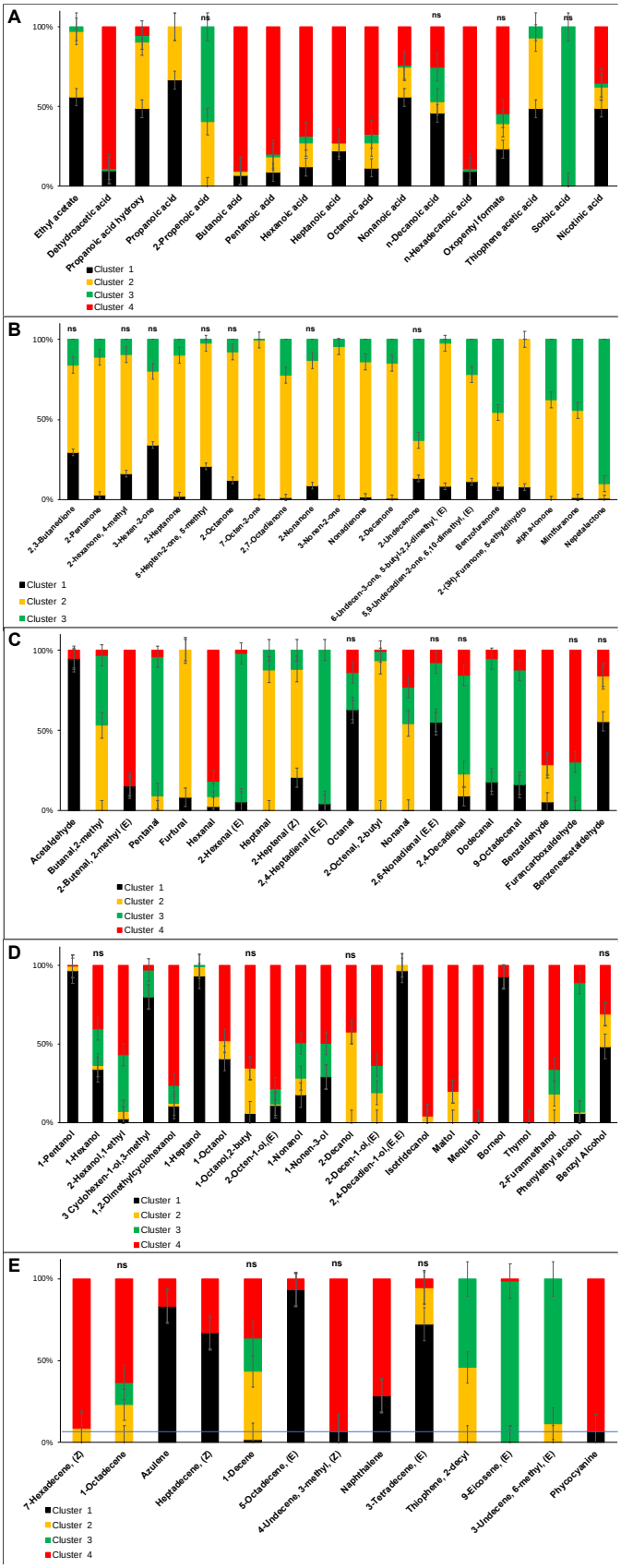


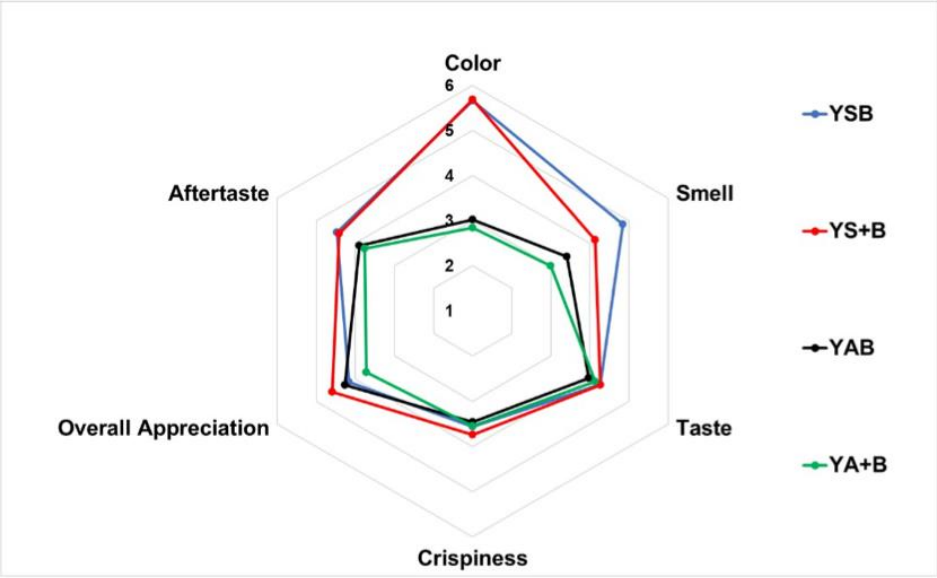
Figure S1. Heatmap of relative quantification by SPME GC-MS of means of VOCs of not fermented samples. Pearson dendrogram and complete linkage on independent variables. FA = Flours of Algae; FM = Flours of Maize; FR = Flours of Rice; AL, AX, AY = Algae type not fermented doughs: SL, SX, SY = Standard type not fermented doughs.

CASE STUDY 2: Bread fortified by spirulina



Supplementary Figure 2. K-means ($p<0.01$) of VOCs on samples. A) Organic acids; B) Ketones; C) Aldehydes; D) Alcohols; E) Alkenes. The cluster legends can be found from Figure 3 to Figure 7.

CASE STUDY 2: Bread fortified by spirulina



Supplementary Figure 3. Spider chart of two independent consumer's tests. Blue plot = YSB (ST bread fermented 6 h by yeast); red plot = YS+B (ST sourdough bread); black plot = YAB (AT bread fermented 6 h by yeast); green plot = YA+B (AT sourdough bread). 0 = unacceptable; 6 = excellent.

5.2. Prebiotic potential of Spirulina bread throughout an in vitro gut model

(In preparation as invited for submission in International Journal of Food Microbiology for the Special Issue of 27th International ICFMH Congress Food Micro 2022)

5.2.1. Introduction

Target audience for GF foods stretches beyond coeliac sufferers. In 2015, only 9% of US GF consumers followed a GF diet due to celiac disease, while others just did it believing it is healthier. Thus, it is necessary to know the impact of such food products on healthy subjects.

Murine model investigations are still widely used for gut microbiota studies. However, due to differences in human physiology and gut microbiota (Hugenholtz and de Vos, 2018) but also due to the incentive for in vitro approaches by the application of European 3R (Replacement, Refinement and Reduction) principle rules (Prescott and Lidster, 2017), in vitro colonic models mimicking the human physiological conditions, including gut microbiota, represent powerful alternative tools.

In this work we compared two formulations of GF bread, with and without *Arhtrospira platensis* powder (AP) for protein enrichment, obtained by sourdough and plain (control) fermentations. Comparison was made after gastro-duodenal digestion and proximal colonic fermentation performed for a short-term experiment on MICODE (Multi-Unit In Vitro Colon Model), employing donations from healthy individuals. The aim is to highlight and compare the impact of formulation and process of the breads on the human colon microbiota, throughout microbiomics (qPCR and 16S MiSeq) and metabolomics (SPME GC-MS).

5.2.2. Materials and methods

5.2.2.1. Human Colon Microbiota

HCM was obtained from the stools of two healthy volunteers, one male and one female, aged between 30 and 45 t. The volunteers were adult not consuming antibiotics, nor pre- or probiotic supplements 3 months prior to the experiment, normal weight, non-smokers, and with no history of chronic

gastrointestinal disorders. Fecal samples were donated two times (with an interval of seven days) for the two biological replicas.
Volunteers were informed of the purposes and procedures of the study and provided their written informed consent, in accordance with the Ethics Committee of the University of Bologna.
Fecal samples were collected, and processed as previously described (Nissen et al., 2021a; Nissen et al., 2021b).

5.2.2.2. Materials

Chemicals, solvents, and enzymes for batch culture fermentation were of the highest analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), and Carlo Erba Reagents (CEDEX, Val de Reuil, FR), unless otherwise stated. Reagents for molecular biology and kits for DNA extraction or purifications were purchased from Thermo Fisher Scientific (USA).

5.2.2.3. Experimental samples and controls

Experimental gluten free algae-enriched and control breads, traditionally processed (6 h of leavening with baker's yeast) and processed by sourdough (24 h of leavening with Baker's yeast and a mix of lactic acid bacteria) were previously prepared and characterized (Chapter 3.3.1.). From this previous characterization, it has emerged that the algae-enriched breads exert a strong prebiotic activity by means of culturomics. The gluten free bread samples tested in the present work were: AS = Algae-enriched breads sourdough processed; AT = Algae-enriched breads traditionally processed; CS = Control breads sourdough processed; CT = Control breads traditionally processed.

5.2.2.4. In vitro digestion and fermentation

Gluten free algae-enriched and control breads were digested in vitro with the INFOGEST protocol (Minekus et al., 2014). Digestive enzymes were deactivated and the digestates were then stored at -80 °C. Prior to in vitro colonic fermentation, the digestates were thawed and gently centrifuged to precipitate the denser portion.
Short-term batch proximal colon fermentations were conducted for 24 hours in independent vessels using an in vitro colon model, MICODE (Nissen et al., 2021a; Nissen et al., 2021b). The preparation of the experiments was made according to published procedures (Connolly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021a; 2021b). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (BM) (Connoly et al., 2012; Koutsos et al., 2017; Diotallevi et al., 2021) and left running to reach and maintain the proximal colon

ecological conditions (0.0% of DO2 and pH 6.75). BM contained (per liter): 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K2HPO4, 0.04 g KH2PO4, 0.01 g MgSO4·7H2O, 0.01 g CaCl2·6H2O, 2 g NaHCO3, 2 mL Tween 80, 0.05 g Hemin dissolved in 1 mL of 4 M-NaOH, 10 mL vitamin K, 0.5 g L-cysteine HCl, and 0.5 g bile salts (sodium glycocholate and sodium taurocholate). The medium was adjusted to pH 7.0 before autoclaving and 2 mL of 0.025% (w/v) resazurin solution were added afterwards once the media was cooled. Fermentation vessels were filled aseptically with 90 mL of BM and the bioreactor headplates were mounted, including previously sterilized and calibrated sensors, i.e., pH and DO2 (Dissolved Oxygen) sensors. Anaerobic condition (0.0 – 0.1% w/v of DO2) in each bioreactor was obtained in about 30 min flushing with filtered O2-free N2 through the mounted-in sparger of Minibio reactors (Applikon Biotechnology BV, NL), and was constantly kept over the experiment. Temperature was set at 37 °C and stirring at 300 rpm, while pH was adjusted to 5.75 and kept throughout the experiment with the automatic addition of filtered NaOH or HCI (0.5 M) to mimic the conditions located in the distal region of the human large intestine. Once the exact environmental settings were reached, the four vessels were aseptically injected with 10 mL of fecal slurry (10% w/v of human feces to a final concentration of 1%, w/v) and then four of them independently with 1 g of AT, AS, CT, or CS (to a final concentration of 1%, w/v), while the fifth vessel was set as blank control (BC, basal medium and 1% fecal slurry only). Batch cultures were run under these controlled conditions for a period of 26.55 h during which samples were collected at 3 time points (Baseline, 18, and 24 h). The baseline (BL) was defined on the first pH changes detected by Lucullus (1 read/10 s) via the pH Sensors of MICODE. For this work, the BL was set after 2.02 ± 0.18 h. Sampling was performed with a dedicated double syringe filtered system (Applikon Biotechnology BV, NL) connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a close control, monitoring and recording of fermentation parameters the software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) was used. This also allowed to keep the stability of all settings during the experiment. Fermentations were conducted in duplicate independent experiments, using for each a new pool of feces from the same two healthy donors.

5.2.2.5. Pipeline of experimental activities

Parallel and independent vessels for AT, AS, CT, CS, and BC were run for 24 h after the adaptation of the fecal inoculum, defined as the BL. The entire experiment was based on 5 theses and 3 time points (BL, 18 h, and 24 h) in biological duplicate (n = 30). Samples of the different time points were used for qPCR, 16S-rDNA MiSeq Illumina Sequencing, and SPME GC-MS analyses. After sterile sampling of 5 mL of bioreactor contents, samples were centrifuged at 16000 × g for 7 min to separate

the pellets and the supernatants, which were used for bacterial DNA extraction and SPME-GC-MS analysis, respectively. The pellets were also washed twice in PBS prior DNA extraction. Microbial DNA extraction was conducted just after sampling so as not to reduce *Firmicutes* content. DNA samples and SPME-GC-MS samples were then stored at -80 °C prior analyses. Technical replicas of analyses were conducted in duplicate for SPME GC-MS (n = 60), in triplicate for qPCR (n = 90), and in duplicates for the BL plus pooled duplicates for the end points of fermentation for 16S-rDNA metataxonomy (n = 7).

5.2.2.6. Microbiota related analyses

5.2.2.6.1. DNA extraction, amplification and sequencing

DNA was extracted at each time points (BL, 18 h, and 24 h) using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK). Samples from the BL, and the EPs were used for metataxonomy by 16S rDNA MiSeq sequencing (Illumina Inc, San Diego, CA, USA), while samples from the BL and other time points were used for quantitative PCR (qPCR) analyses. Considering metataxonomy, bacterial diversity was obtained by the library preparation and sequencing of the 16S rRNA gene. The following two amplification steps were performed: an initial PCR amplification using 16S locus-specific PCR primers (16S-341F 5′-CCTACGGGNGGCWGCAG-3′ and 16S-805R 5′-GACTACHVGGGTATCTAATCC-3′) and a subsequent amplification integrating relevant flow-cell-binding domains (5′-TCGTCG GCAGCGTCAGATGTGTATAAGAGACAG-3′ for the forward primer and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ for the reverse overhang), and lastly unique indices selected among those available Nextera XT Index Kits were combined according to manufacturer's instructions (Illumina Inc, USA). Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, USA). In addition, libraries were quality-tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent technologies, Santa Clara, CA, USA). Libraries were sequenced in a MiSeq (Illumina Inc, USA) in the paired end with 300-bp read length (Marino et al., 2019). Sequencing was conducted by IGA Technology Service Srl (Udine, Italy).

5.2.2.6.2. Sequence data analysis

Reads were de-multiplexed based on Illumina indexing system. Sequences were analyzed using QIIME 2.0 (Caporaso et al., 2010). After filtering based on read quality and length (minimum quality = 25 and minimum length = 200), Operational Taxonomic Units (OTUs) defined by a 97% of similarity were picked using the Uclust v1.2.22 q method (Edgar et al., 2010) and the representative

sequences were submitted to the RDP classifier (Wang et al., 2007) to obtain the taxonomy assignment and the relative abundance of each OTU using the Greengenes 16S rRNA gene database (McDonald et al., 2011) constantly updated by IGA Technology Service Srl (Udine, Italy). Alpha- and beta-diversity analyses were performed using QIIME 2.0.

5.2.2.7. Volatilome analysis

Volatile organic compound (VOCs) evaluation was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The Solid Phase Micro-Extraction (SPME) GC-MS protocol and the identification of volatile compounds were done according to previous reports, with minor modifications (Nissen et al., 2021a; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Briefly, 3 mL of vessel content or fecal slurry were placed into 10-mL glass vials and added to 10 μL of the internal standard (4-methyl-2-pentanol) to a final concentration of 4 mg/L. Samples were then equilibrated for 10 min at 45 °C. SPME fiber, coated with carboxen-polydimethylsiloxane (85 μm), was exposed to each sample for 40 min. Preconditioning, absorption, and desorption phases of SPME–GC analysis, and all data-processing procedures were carried out according to previous publications (Nissen et al., 2021a; Di Cagno et al., 2011; Casciano et al., 2021). Briefly, before each head space sampling, the fiber was exposed to the GC inlet for 10 min for thermal desorption at 250 °C in a blank sample. The samples were then equilibrated for 10 min at 40 °C. The SPME fiber was exposed to each sample for 40 min, and finally the fiber was inserted into the injection port of the GC for a 10 min sample desorption. The temperature program was: 50 °C for 1 min, then programmed at 1.5 °C/min to 65 °C, and finally at 3.5 °C/min to 220 °C, which was maintained for 25 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were carried out in split-less mode and helium (3 mL/min) was used as a carrier gas. Identification of molecules was carried out by searching mass spectra in the available databases (NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). Each VOC was relatively quantified in percentage (LOD = 0.001 mg/kg)29. All results were expressed as normalized mean values obtained from duplicates in two independent experiments.

5.2.2.8. Data processing and statistical analysis

For the volatilome, one-way ANOVA ($p < 0.05$) was used to determine significant VOCs among the dataset, which included 8000 interactions generated between 171 dependent variables (VOCs) and

60 independent variables (2 technical and 2 experimental replicas of 5 different fermentation treatments; AT, AS, CT, CS, and BC, and 3 different time points; BL, T1 = 18 h, and EP = 24 h).

The significant VOCs representing the total volatilome were processed by Principal Component Analysis (PCA) to distribute the results on a plane and multivariate ANOVA (MANOVA) to address specific contributes by categorical predictors.

For the microbiota, after ANOVA for group comparison (the baseline versus the end point), the significant variables ($p < 0.05$) were selected and the shifts in abundance were calculated as Log2(F/C). Then, post hoc Tukey HSD test ($p < 0.05$) was performed on the raw data to define differences among treatments. The microbiota at the endpoint was analyzed as a pool of DNA of the biological replicas for each case, while at the baseline as a pool of the four cases.

Normalization of datasets was performed with the mean centering method. Statistics and graphics were made with Statistica v.8.0 (Tibco, Palo Alto, CA, USA).

5.2.3. **Results**

5.2.3.1. Quality controls for the validation of MICODE protocol

To validate the MICODE experimental approach in the version of fecal batch of the human proximal colon, we chose to monitor and check some parameters as quality controls (QC) related to metabolites and microbes at the end of fermentations, and in comparison, to the baseline. QCs for microbiomics adopted were relative to alpha diversities, that were maintained similar throughout the experimentation. For example, at the EPs, the Chao1 Index, which reflect the microbiota richness, indicated more than 400 OTUs and the Good's Index, which reflects the ecological diversity within rare taxa did not change significantly confirming the capacity to simulate a healthy in vivo condition for 24 h and indicating enough support to the growth of rare or less representative species. Also, the presence of *Archea (e.g., Methanobrevibacter smithii*), which is extremely sensible to oxygen content (Samuel et al., 2007), was retained in the BC vessel from the BL to the EP, indicating that the environmental conditions were strictly maintained.

About the volatilome, we evaluated some stool-related compounds, namely urea, 1-propanol, and indole, that were adsorbed at the same retention times spreading across the complete chromatogram and were similarly quantified for each GC/MS analysis.

5.2.3.2. Changes in bacterial alpha and beta diversities

The microbiota diversity indices were analyzed to study the impact of AP breads on colonic microbial population, to assess population's stability during fermentation, and to compare the microbiota of their vessels to that of other bioreactors (**Figure 1**).

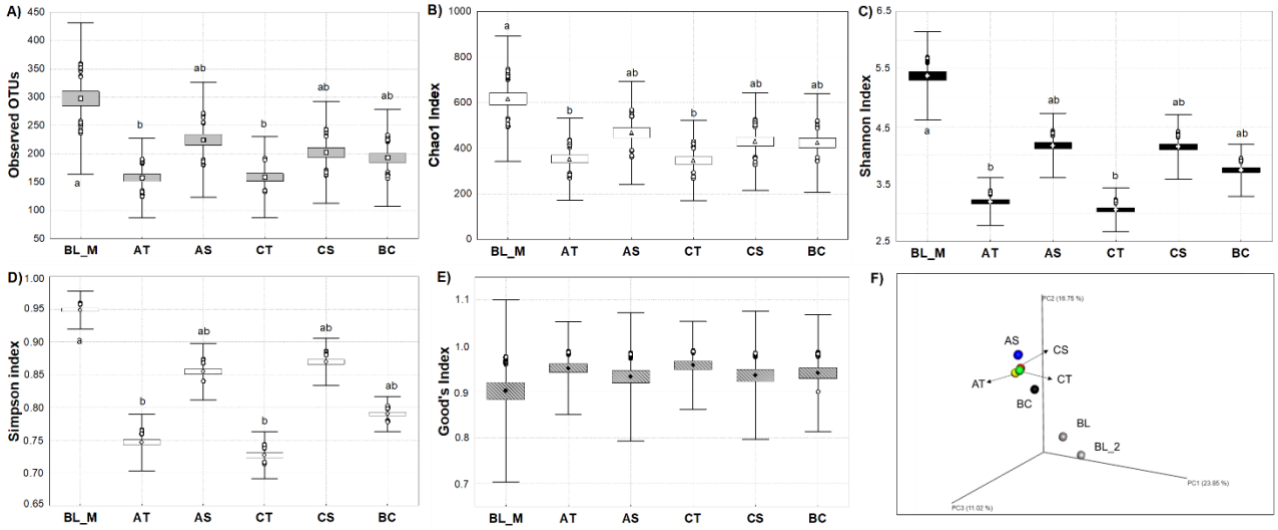


Figure 1. Ecological diversities representing the baseline and at the end points of human colon microbiota after in vitro digestion and fermentation of AP and control gluten free breads. A) Observed OTUs representing richness; B) Chao1 Index representing abundance; C) Shannon Index representing evenness; D) Simpson Index representing dominance; E) Good's Index representing rarity; F) Bray Curtis PCoA of Beta Diversity representing differences among samples. BL_M = Baseline Mean; AT = Algae bread Traditional AT = Algae bread Traditional; AS = Algae bread Sourdough; CT = Control bread Traditional. CS = Control bread Sourdough = CS; BC = Blank Control. Different letters indicates statistical significance.

The baseline of value was compared to the endpoints of different breads fermentations. It is undisputable that abundances and richness diminished over time in the in vitro simulation of any sample, because no supplementation was considered, although not all samples significantly modulated the microbiota. For example, richness (**Figure 1A**) and abundance (**Figure 1B**) were significantly lower at the EPs ($p < 0.05$) just for the traditional baked breads (AT or CT), while were not significant for sourdough processed breads (AS or CS), nor for the BC ($p > 0.05$). Evenness and dominance (**Figure 1C and 1D**) were reduced after fermentation of any sample, but significantly just for AT and CT ($p > 0.05$). These results indicated that sourdough process in comparison to the traditional one affected less the population diversities. The Good's index (**Figure 1E**) remained unchanged after any fermentation, indicating the effectiveness of the in vitro model adopted, that was able to maintain in culture for the whole period of experimentation even rare bacterial taxa. Considering the beta diversity, the Bray Curtis PCoA (**Figure 1F**) has drawn larger differences as an effect of time (BL vs EP of fermentations), than as an effect of matrix (AT, AS, CT, CS). Still this

outcome is a positive feature for the effectiveness of the in vitro model, that indicated different diversities among bioreactors after fermentation.

5.2.3.3. Changes in taxa abundances at the phylum level

The total sequence reads used in this study were classified into eight phyla and one unassigned (**Table 2**). In any tested sample, the core microbiota at the BL was represented by four taxa: three with a relative abundance higher than 10% (*Firmicutes, Bacteroidetes,* and *Actinobacteria*) and one lower than 5% (*Proteobacteria*). Anyhow, *just Firmicutes*, *Bacteroidetes*, and *Proteobacteria* underwent significative changes in comparison to the BL ($p < 0.05$). At this taxonomic level after fermentation the changes were different among the substrates just for *Bacteroidetes*, and *Proteobacteria*, relatively to control samples and the BC.

Table 2. Metataxonomy of Abundances and Changes of Human Colon Microbiota*.

OUT ID#	**Abundance**	**Changes at the end points**					**ANOVA****	
	RQ (%) ± SD	Log_2 (F/C)					*p value*	*-Log_{10}(p)*
	BL	**AS**	**AT**	**CS**	**CT**	**BC**		
Unclassified	0.001 ± 0.001	3.39^{b}	4.59^{ab}	4.91^{a}	3.88^{ab}	4.66^{ab}	0.021671	1.664121
Bacteria;Other	0.147 ± 0.093	1.39	-0.18	-0.37	0.26	-0.21	0.685445	0.164027
Actinobacteria	10.906 ± 6.188	0.79	-0.29	-0.25	0.61	-0.79	0.826103	0.082966
Bacteroidetes	30.082 ± 1.067	-1.78^{ab}	-1.63^{ab}	-2.38^{b}	-0.73^{a}	-1.72^{ab}	0.002367	2.625802
Cyanobacteria	0.012 ± 0.009	nd	nd	nd	nd	-3.52	0.017529	1.756243
Firmicutes	55.200 ± 8.468	-0.71^{ab}	-0.72^{ab}	-0.66^{a}	-0.73^{ab}	-1.11^{b}	0.002834	2.547600
Fusobacteria	0.003 ± 0.004	nd	nd	nd	-0.48	nd	0.200410	0.698081
Proteobacteria	3.638 ± 1.312	3.36^{b}	3.72^{abc}	3.78^{ac}	3.09^{b}	3.75^{a}	0.003981	2.400008
Verrucomicrobia	0.010 ± 0.012	3.15	-1.96	-1.32	1.36	nd	0.637886	0.195257

*Sequencing of each sample was obtained from pooled DNA of two different experiments, except for the baseline. The two experiments were performed with two sets of pools of colon microbiotas from three healthy donors; # Constructed from Biome files; **ANOVA model for group comparison on time effect. -*Log_{10}(p)* = Significance of Log_2 (F/C). RQ = Relative Quantity; BL = Baseline; AS = Algae bread sourdough; AT = Algae breads traditional; CS = Control bread sourdough = CS; CT = Control bread traditional.

5.2.3.4. Metataxonomy at the family and species level

The OTUs were filtered up to a cutoffs of 0.001% and among 65 families assigned OTUs in the entire dataset, 33 was the mean number at the BL. After fermentation, just AS was the substrate capable to retain them all, while the other substrates reduced the number of different family taxon.

193 OTUs were constructed and assigned to microbial taxa (cutoffs 0.001%) at the BL. Of these, 171, 146, 152, 150, and 166 were identified at the EPs of fermentation of AS, AT, CS, CT, and BC, respectively (**Figure 2A**). At the EP, AS was the substrate that shared most of the taxa found at the

BL. Also, from the Intersection Map, the sourdough processed substrates shared more taxa with the BL, than the traditional ones. Regarding the exclusive presence of taxa that are relative to each substrate fermentation, AS has the highest number, more than the double in respect to AT and CT, and almost four times than CS (**Figure 2B**). Among these taxa, AS was characterized by important *Ruminococcus albus*, that is a beneficial commensal, known to be negatively correlated to IBD (Inflammatory Bowel Disease) (Nagao-Kitamoto & Kamada, 2017), but also to harmful *Desulfovibrio*, that is a sulfate reducer culprit of colitis (Rowan et al., 2010). AT at the EP of fermentation was characterized by the exclusive presence of beneficial *Lactobacillus crispatus* (Patrignani et al., 2020), but also to that of *Streptococcus sanguinis*, which is associated to possible infections (Martini et al., 2020). CS was characterized by the exclusive presence of *Streptococcus infantis*, which is a commensal in the oral cavity, but an opportunistic when is transferred to other niches (Zhou et al., 2020). CT was characterized by the exclusive presence of beneficial *Lactobacillus mucosae* (Bagon et al., 2021), but also by that of several opportunistic *Proteobacteria*, such as *Citrobacter spp.* (Liu et al., 2020). Lastly, it is important to note that *Methanobrevibacter smithii* was an exclusive species found in the BC, that is considered as a QC of our colonic model ecological settings, as this taxon is highly sensible to oxygen (Samuel et al., 2007).

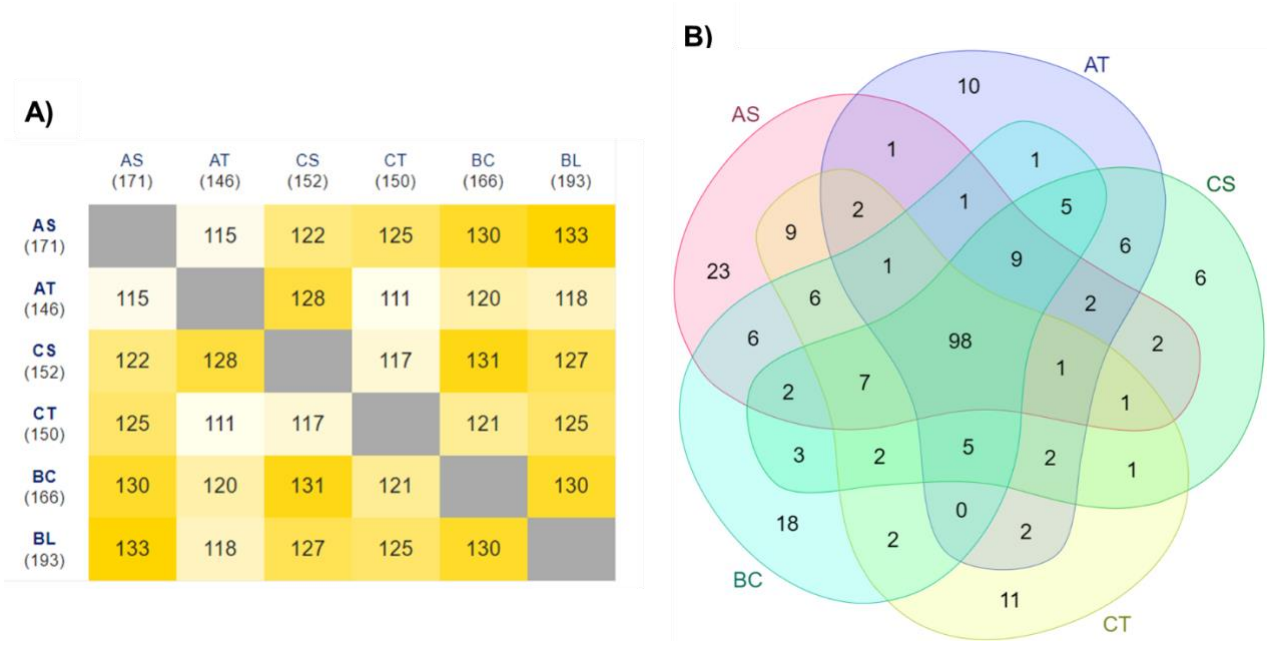


Figure 2. Pair Wise Intersection Map and Venn Diagrams representing the microbiome lists at species level of human colon microbiota after in vitro digestion and fermentation (end points) of algae-enriched gluten-free breads and control gluten-free breads in respect to the baseline. BL = baseline; AS = Algae bread Sourdough; AT = Algae breads Traditional; CS = Control bread Sourdough = CS; CT = Control bread Traditional.

5.2.3.5. Changes in taxa abundances at family and species levels

The results relative to AS substrate fermentation indicated positive outcome as significant changes at family level, like important overrepresentation of beneficial and commensal taxa, as *Bifidobacteriaceae* (> 6.679 folds) and *Verrucomicrobiaceae* (> 4.354 folds), but also significant underrepresentation of some opportunistic taxa. In contrast, a negative outcome is that of significant underrepresentation of renown families as butyrate producers, such as *Lachnospiraceae* (< 2.73 folds) and *Ruminococcaceae* (< 4.44 folds)

At species level there were important overrepresentation of beneficial Bifidobacterium bifidum (> 5.78 folds) and, immunostimulant *Lactobacillus gasseri* (> 8.55 folds), bacteriocin producers *Enterococcus durans* (> 8.83 folds), and postbiotic producer *Akkermansia muciniphila* (> 5.62 folds). Also, still considered as a positive result, the taxa underrepresented in comparison to the BL were those of several opportunistics and metabolic syndrome associated, as *Ruminococccus torques* (< 9.42 folds), *R. gnavus* (< 5.37 folds), *Dialister invisus* (< 2.62 folds), but, as a negative result, also that of commensal *Bacteroides vulgatus* (< 3.43 folds) and *Roseburia faecis* (< 6.23 folds).

The results relative to AT substrate fermentation indicated positive outcome as significant changes at family level, likely important overrepresentation of *Enterobacteriaceae* (> 7.09 folds) and *Clostridiaceae* (> 4.96 folds), also an underrepresentation of some opportunistic taxa is evidenced, like *Pasteurellaceae* (< 4.81 folds), but indicated also negative feature as the reduction of other commensals, as *Ruminococcaceae* (< 8.65 folds) and *Lachnospiraceae* (< 3.06 folds). At species level there are important overrepresentation of beneficial immunostimulant *L. gasseri* (> 6.98 folds) and bacteriocin producers *E. durans* (> 1.85 folds), but also that of opportunistic *Escherichia alberti* (>6.62 folds) and *Clostridium butyricum* (> 10.05 folds). As a positive effect, the opportunist *Blautia* was significantly underrepresented (< 2.50 folds), but in contrast *A. muciniphila* was reduced (< 2.79 folds).

From the recipient results it is evidenced that in general GF breads are not modulating positively the microbiome of not celiac subjects, except when the sourdough process and the enrichment with algae are applied.

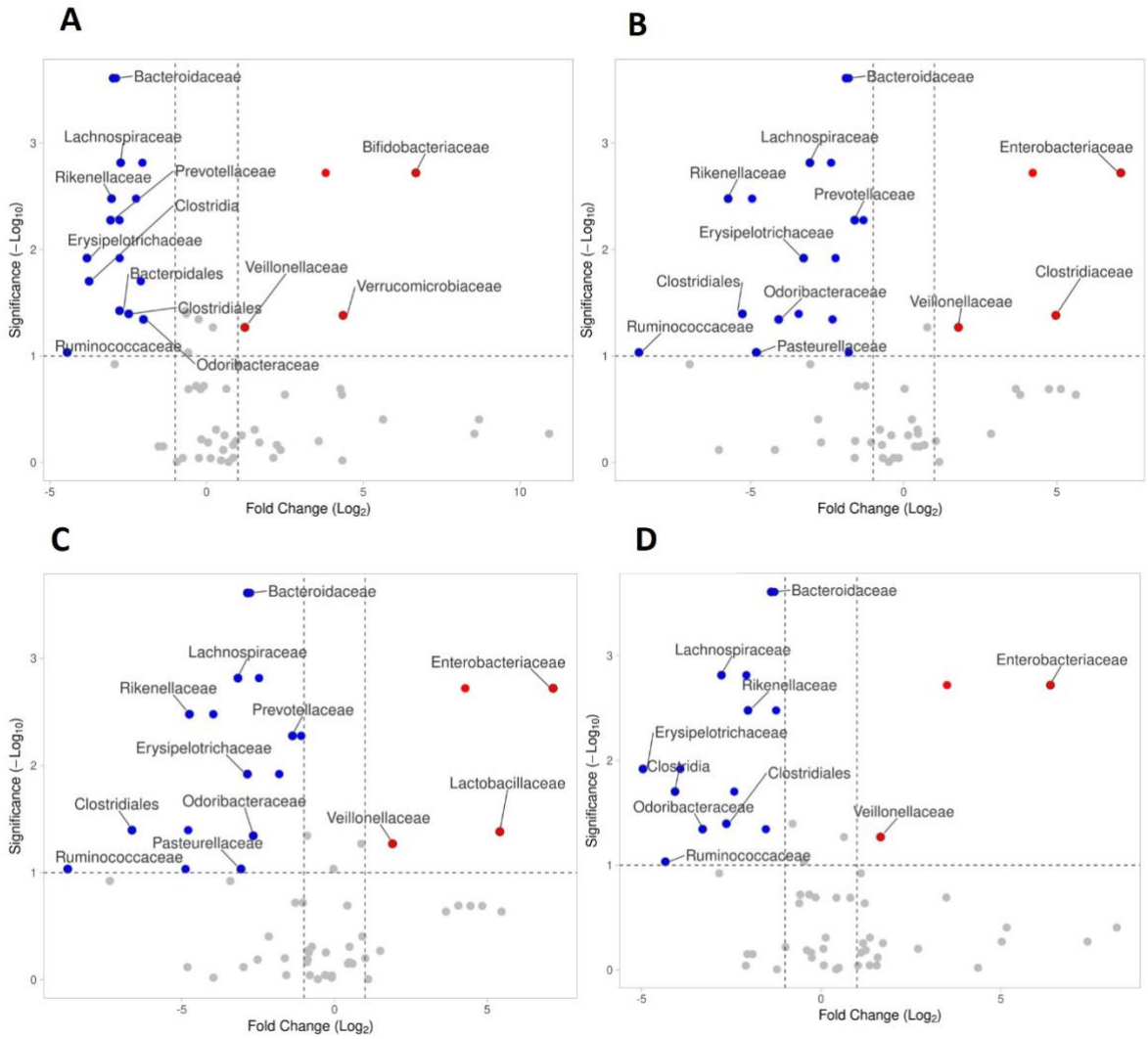


Figure 3. Volcano plots representing changes of human colon microbiota at family level after in vitro digestion and fermentation of AP and control gluten free breads expressed as Log2(F/C) in respect to the baseline. Red dots = overrepresented variables; Blue dots = underrepresented variables; Grey dots = unchanged variables A) Algae bread sourdough = AS; B) Algae breads traditional = AT; C) Control bread sourdough = CS; D) Control bread traditional = CT

The results relative to CS substrate fermentation (Figure 3C) indicated that at family level there are important overrepresentation of *Enterobacteriaceae* (> 7.15 folds) and *Lactobacillaceae* (> 5.41 folds) and significant underrepresentation of some opportunistic taxa, as *Pastereullaceae* (< 3.06 folds).

At species level significant overrepresentation of *Lactobacillus gasseri* (> 6.67 folds) and *Escherichia alberti* (> 6.63 folds) were confirmed, also underrepresentation of several opportunistic *Clostridiales*, but also that of essential *Faecalibacterium* (< 6.35 folds).

The results relative to CT substrate fermentation (Figure 3D) indicated that at family level there are important significant overrepresentation of *Enterobacteriaceae* and significant underrepresentation

opportunistic *Clostridia*, but also of important *Ruminococcaceae*. At species level significant overrepresentation of *Escherichia alberti* (>5.99 folds) was confirmed, also underrepresentation of *Clostridium butyricum* (< 2.18 folds).

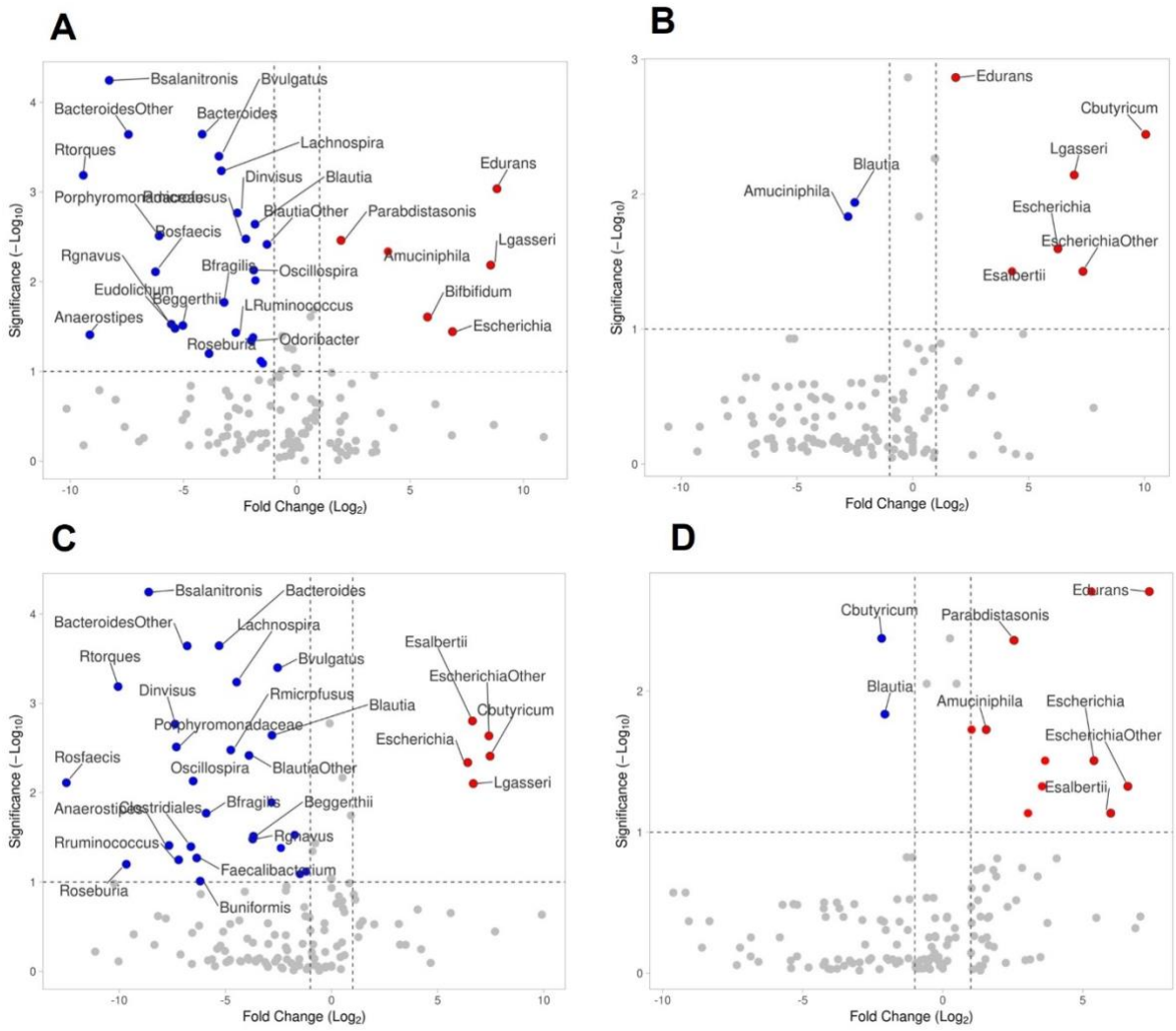


Figure 4. Volcano plots representing changes of human colon microbiota at species level after in vitro digestion and fermentation of AP and control gluten free breads expressed as Log2(F/C) in respect to the baseline. Red dots = overrepresented variables; Blue dots = underrepresented variables; Grey dots = unchanged variables A) Algae bread sourdough = AS; B) Algae breads traditional = AT; C) Control bread sourdough = CS; D) Control bread traditional

5.2.3.6. Volatilome analysis through SPME GC/MS

Through SPME GC-MS, among 30 duplicated cases (n = 60), 171 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). From the Pair Wise intersection Map (**Figure 5A**) on average, 125 were relatively quantified at the BL, while 108, 101, 93, and 95 were quantified during the 24 h of

experiments at different timepoints for AS, AT, CS, and CT, respectively Also 74 VOCs were averagely found during fermentation in the BC. Regarding the Venn Diagram is interest to mention that some of the exclusive VOCs found in AS were Caryophillene, m-Cymene-5-tert-butyl, and Beta-Alanina, while some of those found in CS were 3-Tridecene (Z) and Furan, 2-methoxy.

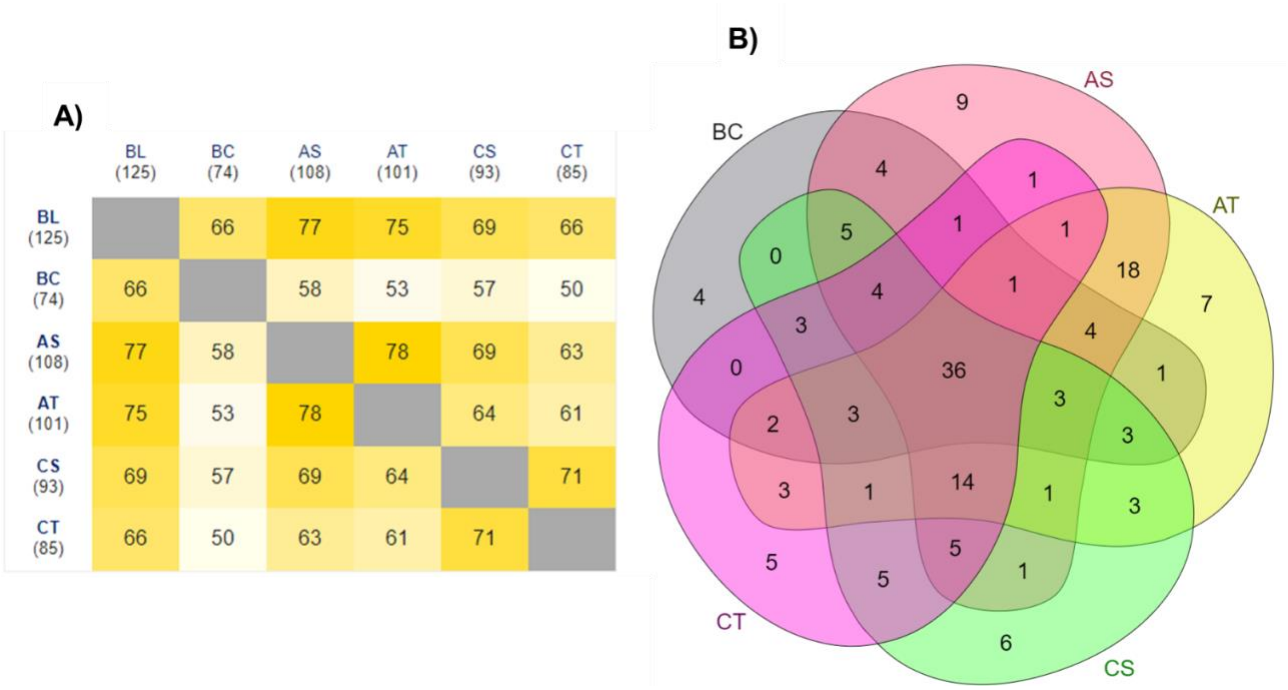


Figure 5. Pair Wise Intersection Map and Venn Diagrams representing the volatilome list of VOCs of human colon microbiota after in vitro digestion and fermentation (end points) of algae-enriched gluten-free breads and control gluten-free breads in respect to the baseline. BL = baseline; AS = Algae bread Sourdough; AT = Algae breads Traditional; CS = Control bread Sourdough = CS; CT = Control bread Traditional.

Principal Component Analysis (PCA) and targeted MANOVA ($p < 0.01$) was achieved to address the specific contributes to VOCs production by the independent variables. Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing one chemical class to another. In these datasets T1 cases are also considered.

A PCA of 22 statistically significant low molecular alcohols distributed cases on the plot, separating fermentation with algae breads to that of control breads, and the BC from each other and from the baseline (**Figure 5**). By MANOVA the main descriptors of algae enriched breads were 1,6-Octadien-3-ol, 3,7-dimethyl, 2-Decen-1-ol, (E), and trans-2-Undecen-1-ol, chiefly produced by the sourdough process at the EP. In contrast descriptors of control breads were 2-Heptanol, 3-methyl, Ethyl alcohol,

Isopropyl alcohol, and 1-Propanol, mainly produced by the sourdough process at the EP of fermentation. Thus, alcohols derived from the matrix are including Geraniol and olefins as descriptors of AS, while alcohols derived mainly from colonic fermentation are descriptors of CS. Geraniol is known to own an antioxidant nature and it is originally found in spirulina enriched breads (Casciano et al., 2021).

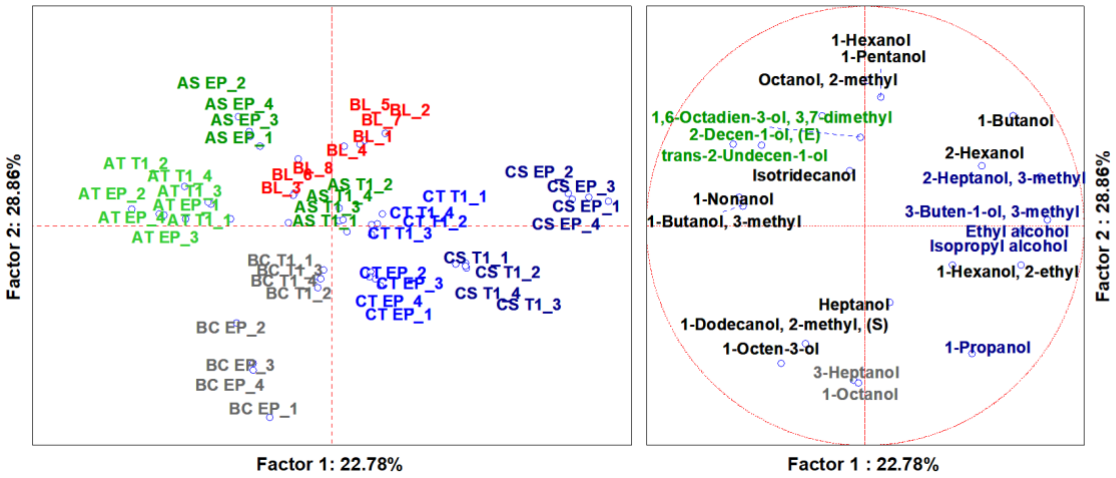


Figure 5. Principal Component analysis and Multivariate Analysis of Variance of low molecular alcohols VOCs.

A PCA of 20 statistically significant high molecular alcohols distributed cases on the plot, separating fermentation with AS, AT, control breads, and the BC from each other and from the BL (**Figure 6**). From our results, the group of cases of AS was set as the most distant to BL of fermentation. The main descriptors of fermentation with AS were mainly complex alcohols ($p < 0.01$), such as N-(Cyanoethyl)-pyrrole, Benzyl alcohol, and Phenylethyl alcohol mainly produced at the EP ($p < 0.01$) while those for control breads were Phenol, p-tert-butyl and 7-Tetradecanol, mainly produced by the sourdough process either at T1 or EP ($p < 0.01$). The main descriptor of alcohol production from BC samples were instead skatole and Thiazole largely produced at the EP ($p > 0.01$). Thus, AS had the exclusive sign of bioactive such as N-(Cyanoethyl)-pyrrole, that is a catabolic block of Phycocyanin, the top antioxidant compound present in spirulina (Casciano et al., 2021). Differently, long chain fatty alcohols derived mainly from colonic fermentation, but also food contaminants are descriptors of CS.

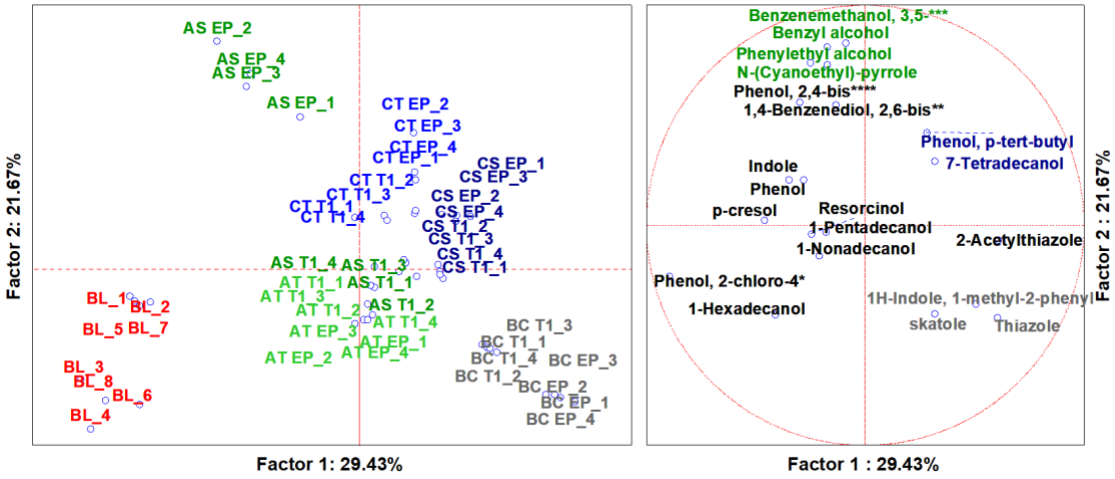


Figure 6. Principal Component analysis and Multivariate Analysis of Variance of high molecular alcohols VOCs.

A PCA of 15 statistically significant aldehydes distributed cases on the plot, separating fermentation with AS and CS distant to each other and distant from the BL and the BC (**Figure 7**). The main descriptor of fermentation with AS was 2-Nonenal, (E), mainly produced at the EP ($p < 0.01$). The main descriptors of fermentation with CS were Butanl-2-methyl and Heptanal ($p < 0.01$), while those of the BC were Benzaldehyde, 2,4-dimethyl and 2-Furancarboxaldehyde either produced at T1 and EP ($p < 0.01$). Aldehydes are a result of microbial fermentation and lipid oxidation. Certain aldehydes are health-promoters, like 2-Nonenal, (E) that was reported to limit the growth of several intestinal pathogens at a very low concentration (Zhang et al., 2020), while most are detrimental, being cytotoxic at a low threshold, such as Benzeneacetaldheyde (Zhang et al., 2020). Otherwise, 2-Nonenal that in our dataset is a descriptor of colonic fermentation of AS is reported to be an oxidation product of fish oils and so far, its presence could be toxic for the host.

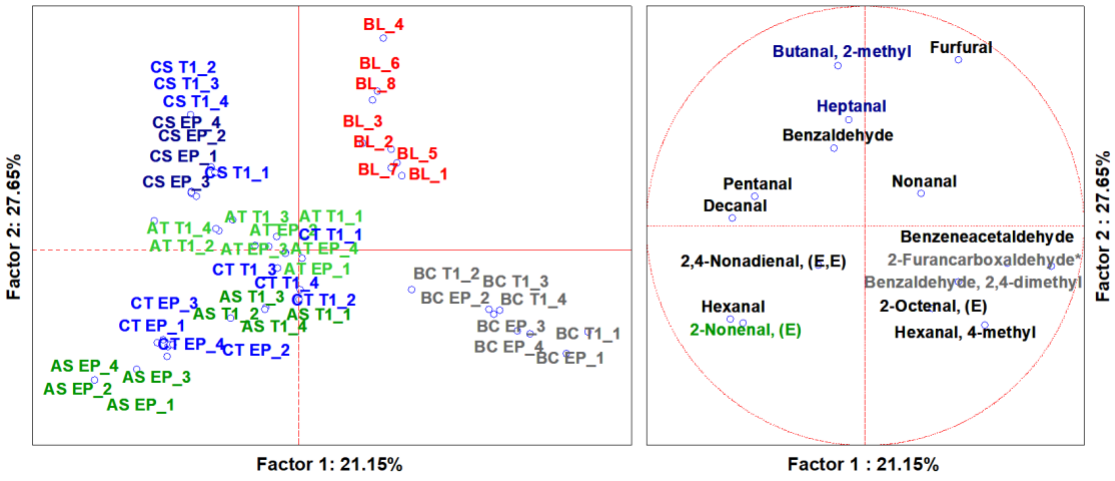


Figure 7. Principal Component analysis and Multivariate Analysis of Variance of aldehydes VOCs.

A PCA of 23 statistically significant other VOCs, related to alkenes distributed cases on the plot, separating the substrates from each other and from the baseline (**Figure 8**). The main descriptor of fermentation with AS were Naphthalene octahydro, 2,4-dimethyl-heptene, and m-Cymene, 5-tert-butyl, and Caryophillene mainly produced at the EP of fermentation ($p < 0.01$). While the main descriptors of CS were 3-Tridecene, (Z) and 3-Pentene, 2,3-dimethyl, either produced at T1 or EP. Caryophillene and m-Cymene, 5-tert-butyl are potent health-related terpenes3 and the features observed indicate that the descriptors of AS were not subject to fermentation and thus their bioactivity was preserved from the food matrix. Thus, while bioactives and antioxidant terpenes are descriptors of colonic fermentation of AS, Olefins and food additives are retained in higher portion after digestion and colonic fermentation of controls as are descriptors of CS.

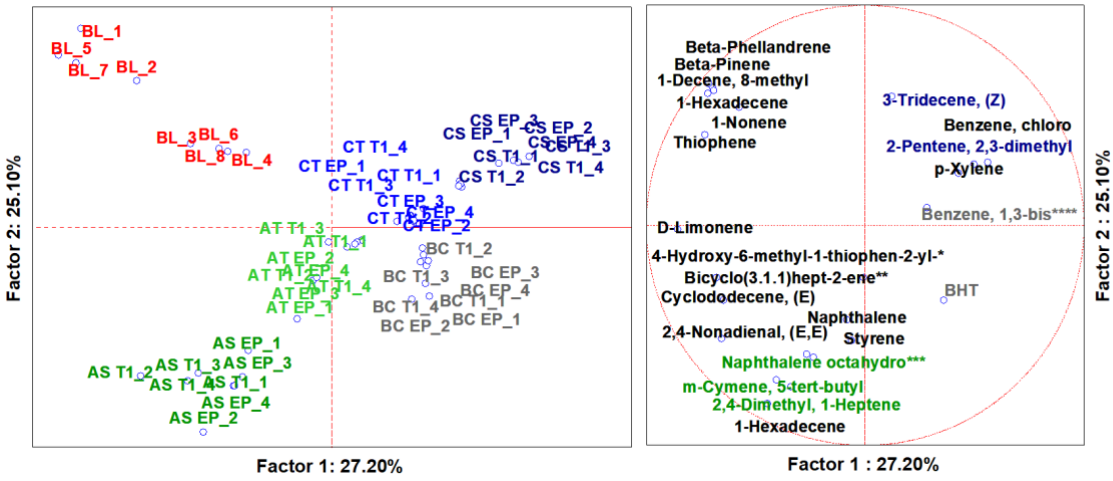


Figure 8. Principal Component analysis and Multivariate Analysis of Variance of alkenes VOCs.

5.2.4. **Conclusions**

The impact of control gluten free breads on the colon ecology of not-celiac origins is not prebiotic, but is inducers of opportunistic overgrowth, raising the numbers of *Enterobacteriaceae*.

This effect is mitigated by the process technology. In fact, when a sourdough fermentation is applied to the breads, it results in a positive modulation of the colon microbiota in respect to the beneficial taxa. Based on this scenario, our results indicates that the combination of sourdough fermentation and Algae breads is able to exert a prebiotic effect, fostering some beneficials and limiting some opportunistics, and also permit an higher retention of bioactives and their microbial more bioaccessible forms.

The next step will be identifying statistical correlations between microbiomic and metabolomic data sets also by means of Machine Learning approach, using a novel K-cliques Multiomic Framework generated by the research team (https://doi.org/10.3390/metabo12080736).

Then to confirm and extend these results experimentations on an in vitro celiac model and long-term model will be necessary.

The results from the in vitro model would serve as a robust foundation for clinical applications on nutritional intervention trial, and also serve as a tool to reduce the animal testing in line with One Health approach.

A harmonization of different in vitro models together with the creation of multi-omics shared data sets would support clinical studies by a deep knowledge of gut-food interactions.

The use of MICODE, a robust and versatile in vitro model, together with multivariate statistics visibly demonstrated a suitable approach to describe the effects generated by GF foods on healthy individuals. Such in vitro approach could be included in a pipeline of experiments where a reduced number of animals for testing is employed, according to the Directive 2010/63/EU and the Regulation (EU) 2019/1010. To fully understand the effect of GF breads on human health of not celiac subjects a diet intervention study is imperative, and the results presented are target-effective and should have robustness for pre-clinical applications.

5.2.5. **References**

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6. CASE STUDY 3: Alternative formualtions to mitigate the nasty impact of commercial salami on colon microbiota

(Submitted to Food Bioscience 06/01/2023)

6.1. Introduction

Salami is a cured sausage consisting of fermented and air-dried meat, typically pork, obtained from a mixture of meat and fat with spices and other ingredients. In general, excessive consumption of processed meat is negatively considered because of its high contents in fat and salt and low contents in bioactive molecules, such as phenolic compounds (Martínez et al., 2014). Despite this, salami is largely consumed around all the world (Blaiotta et al., 2018) and for this reason there is a common interest for an improvement of their nutritional and health properties, e.g., by using probiotic as starter for the fermentation process (Giello et al., 2018), by adding bioactive compounds (dos Santos et al., 2021), by substituting nitrites and nitrates with natural extract (Pini et al., 2020) or by simple nitrites and nitrates withdrawal (Tabanelli et al., 2022). For example, in a study by Pérez-Burillo et al. (2019), the authors added a different type of fiber to salami (citrus fiber, arabinogalactans, and inulin) and evaluated the effect. The results showed that all samples had a higher prevalence of *Bacteroides* in respect to the control, and that the addition of fibers resulted in a reduction of some human intestinal pathogens (Pérez-Burillo et al., 2019).

Among others, a well-known negative characteristic of processed meat is the presence of nitrates and nitrites. The functions of these additives are many, e.g., prevention of lipid oxidation, color maintenance, and microbiological safety by inhibiting pathogens (Majou e Christieans, 2018), but in recent years those additives have been under attack for their capacity to form N-nitrous carcinogenic compounds. In fact, in the human colon amines and amides are deriving from the bacterial metabolism of aminoacids. These could be N-nitrousated in the presence of nitrosylated heme derived from not absorbed residual of red meat (Herrmann et al., 2015; Johnson, 2017; Meurillon e Engel, 2016). The request from consumers of clean labeled foods, minimally processed and with few additives is growing (Majou e Christieans, 2018). The first research on ingredients alternative to nitrates and nitrites resulted in a product with low organoleptic and microbiological quality (Hammes, 2012).

On the other hand, many studies tested some vegetal extracts as a substitute of nitrates and nitrites thanks to their high polyphenol content, known for their antioxidant and antimicrobial properties

(Jiang & Xiong, 2016; Shah et al., 2014; Shan et al., 2009; Pini et al., 2020). Recently, new salami formulations with nitrate-reducing microbial starter cultures and vegetal extracts bringing 0.4 g/kg of bioactive polyphenols to the meat mixture were developed, without affecting negatively the release of fatty acids and the hydrolysis of proteins during digestion (Di Nunzio et al., 2022)., Additionally, these latter salami digestates were even tested for their effect on HT29 cell lines of the human small intestine, showing no difference in respect to controls (Di Nunzio et al., 2022). Nonetheless, none of these studies focused on the effect of such alternative formulations on gut microbiota perturbations.
For this purpose, in this work, an *in vitro* intestinal model of the proximal colon (MICODE – Multi-unit in vitro colon model), was used to mimic the effect of colon microbiota fermentation. The whole pipeline, including protocols, equipment, and data management, previously demonstrated high reliability as resulted by a very high level of control of ecosystem conditions, the maintenance of the original diversity, rarity, and richness of the human gut microbiota such as some *Archaea* and more than 400 different OTUs (Nissen et al., 2021; Nissen et al., 2021a; Nissen et al., 2022). Therefore, the effects of the replacement of nitrates/nitrites with plant extracts in salami on gut microbiota were evaluated in MICODE through shifts of the microbial populations by qPCR and their volatile metabolites (VOCs) by SPME-GC-MS, while data management approach allowed to explore the correlations among bacterial taxa and beneficial or detrimental metabolites.

6.2. Materials and methods

6.2.1. Experimental samples and controls

Four different salami formulations were tested. For all the formulations, the salami mixture consisted of lean muscle tissue (75%) and minced bacon (25%). The meat was weighed, cut into small pieces, ground in a meat mincer (∅ = 6 mm plate), and then mixed with salt (2.5%), dextrose (0.2%), ascorbate (0.05%) and natural flavours. The positive control formulation (CNO_2) was added with sodium nitrite, potassium nitrate and nitrate-reducing microbial starter cultures (MSC). MSC (Chr. Hansen, S.p.A., Parma, Italy) contained lactic acid bacteria and nitrate-reducing coagulase negative *Staphylococcaceae* and was inoculated as common manufacturing practices to properly drive the fermentation phase and to promote the development of aroma during the ripening phase.
Two innovative formulations not containing nitrites were prepared: the first (SA) was added with MSC and sodium ascorbate (0.3%); the second (SMA) was added with MSC, sodium ascorbate (0.3%), and plant extracts from grapeseed, green tea and, olive (Indena S.p.A., Milan, Italy), characterized according to their total polyphenols content to provide 0.4 g/kg of bioactive polyphenols to the meat mixture. Finally, the negative control (CO) was prepared with neither MSC

nor additives (nitrite, polyphenols and, ascorbate). The formulations of salami and a detailed description of processing are reported elsewhere (Di Nunzio et al., 20222; Saccani et al., 2023).

6.2.2. Experimental Workflow

Briefly, salami samples were processed for gastro-duodenal digestion as described in Di Nunzio et al. (2022), then the digestates were transferred in MICODE *in vitro* colon model for proximal colonic fermentation, using human colon microbiota (HCM). The shifts of the colon microbiota and its metabolites that occurred with fermentation were then studied.

6.2.3. Human Colon Microbiota

HCM was obtained from the stools of three lean healthy individual. The volunteers were adults, not consuming antibiotics, pre- or probiotic supplements in the 3 months prior to the experiment, non-smokers, and with no history of chronic gastrointestinal disorders (Connoly et al, 2012; Nissen et al., 2021; Arnal et al., 2021). Volunteers were informed of the purposes and procedures of the study and provided their written informed consent, in accordance with the Ethics Committee of the University of Bologna.

Human stools were collected by volunteers in a dedicated sterile container, placed in an anaerobic jar with oxygen catalyst (Oxoid, Thermo Fisher Scientific, USA), transferred to the laboratory, and processed within 2 h. HCM was obtained by homogenizing 2 g of each donation in 54 mL of pre-reduced phosphate buffered saline (PBS) (Wang et al, 2020; Nissen et al, 2022).

6.2.4. In vitro gut model

The *in vitro* gastro-intestinal digestion was carried out on salami samples by applying the INFOGEST protocol (Minekus et al., 2014). At the end of the intestinal phase, an aliquot of sample was withdrawn, centrifuged (10000g, 10 min, 4 °C) and the supernatant stored at -80 °C for further analysis, whereas the remaining material (the denser emulsion) was subjected to the in vitro colonic fermentation trials as described below. Digestions were carried out in triplicate.

Proximal colonic fermentations were conducted for 24 hours in independent vessels using an in vitro gut model, MICODE (Nissen et al., 2021; Nissen et al., 2021a). The preparation of the experiments was made according to published procedures (Connoly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021a). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (Connoly et al., 2012; Nissen et al., 2021). Once the proximal colon condition was reached, each vessel was aseptically loaded with 10 mL of independent mixtures including fecal slurry (10% w/v of human feces in O2 reduced PBS) and 1 g of in vitro digested

Salami with ascorbate (SA), Salami with ascorbate and plant extracts (SMA), control with no nitrite (CO), commercial control with nitrate (CNO_2) at a final concentration of 1% (w/v). A fourth vessel was set as blank control (BC) (basal medium and 10% fecal slurry with 1% of digestive enzymes). Batch cultures were run under controlled conditions for a period of 25.52 h including the baseline (BL) (for these experiments set at 1.52 ± 0.18 h) as described in Nissen et al. (2021a). Sampling was performed as reported in Nissen et al. (2021).

6.2.5. Experimental set up and pipeline of activities

Parallel and independent vessels for SA, SMA, CNO_2, CO, and a blank control (BC) were run for 24 h after the adaptation of the fecal inoculum, defined as the baseline (BL). The entire experiment consisted of 5 cases biologically duplicated (SA, SMA, CNO_2, CO, and BC) (n = 10), 3 time points (BL = 1.52 ± 0.18 h), T1 = 18 h, and EP = 24 h) (n = 30) in technical duplicates for GC-MS (n = 60) and technical triplicates (n = 90). Samples of the different time points were used for qPCR and SPME GC-MS analyses. After sterile sampling of 4.2 mL of bioreactor contents, samples were centrifuged at 17000 × g for 7 min to separate the pellets and the supernatants, which were used for bacterial DNA extraction and SPME-GC-MS analysis, respectively. Specifically, microbial DNA extraction was conducted just after sampling so as not to reduce Firmicutes content. After, separation of the pellets from the supernatants, the pellets were washed twice in O_2 reduced PBS to increase the cleaning. DNAs for microbiomics and supernatants for SPME-GC-MS were then stored at -80 °C.

6.2.6. Microbiomics

6.2.6.1. DNA extraction

Bacterial DNA was extracted from the MICODE eluates at each time points, just after sampling; at the baseline, at T1, and EP using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

6.2.6.2. Absolute enumeration of bacterial groups by qPCR

Enumeration of bacterial groups was made by qPCR to evidence changes in the microbiota after fermentation (Tanner et al., 2014; Westfall, Lomis & Prakash, 2018; Tsitko et al., 2019; Nissen et al., 2022a; Tamargo et al., 2022) following previous protocols (Modesto et al., 2011; Nissen et al., 2022; Nissen et al., 2022a). Specifically, the bacterial groups were selected as generally accepted indicators of eubiotic or dysbiotic state of colon microbiota; thereafter, their perturbations may be considered closely correlated (directly or inversely) to the prebiotic potential of foods. 16 different bacterial taxa,

(Table S1), were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA).

6.2.7. Metabolomics

6.2.7.1. Volatilome analysis

Volatile organic compound (VOCs) evaluation was carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 5975 mass spectrometer operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The Solid Phase Micro-Extraction (SPME) GC-MS protocol and the identification of volatile compounds were done according to previous reports, with minor modifications (Nissen et al, 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Identification of molecules was carried out by searching mass spectra in the available databases (NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). Each VOC was relatively quantified in percentage (LOD = 0.001 mg/kg) (Bonfrate et al., 2020).

6.2.7.2. Quantification of main microbial VOCs

In samples before *in vitro* colonic fermentation (BL) (Table S2) the main microbial metabolites related to fermentation of foods were also absolutely quantified in mg/kg with the aforementioned SPME GC-MS approach and the internal standard, but with different cutoffs (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Di Cagno et al., 2011; Nissen et al., 2021; Casciano et al., 2021). For these compounds, samples at T1 and EP were compared to the BL and values were expressed as shifts. Values were computed as follows; i) each single compound was normalized (mean centering method) within its dataset, which included cases from SA, SMA, CNO_2, CO, and BC at different time points; ii) the BL dataset (Table S2) was then subtracted to the fermentation time points; iii) post-hoc analysis was done to compare the sample productions of a single molecule.

6.2.8. Data processing and statistical analysis

For metabolomics, one-way ANOVA model ($p < 0.05$) was used to determine significant VOCs among the raw data of peak's area of the GC-MS chromatograms. The significant VOCs (n = 69) representing the total volatilome of the experiments were analyzed differently; i) the volatilome was relatively quantified, sorted for main chemical classes, and super-normalized, then each dataset was computed for Principal Component Analysis (PCA) to distribute the results on a plane and coupled to Multivariate ANOVA (MANOVA) ($p < 0.01$) (Table S3 and S4) to address specific contributes by

categorical predictors; ii) 9 main VOCs related to microbial fermentation of foods were absolutely quantified and normalized and their BL values were subtracted from T1 and EP values and represented as box plots, including post hoc Tukey HSD test ($p < 0.05$).

For microbiomics, MANOVA ($p < 0.05$) model (categorized for the time points and the treatments) was used to study the shifts in abundance of qPCR values, calculated as $Log_2(F/C)$ (Love et al., 2014). Then, post hoc Tukey HSD test on the raw data ($p < 0.05$) was performed to define differences among treatments or time points. The baselines of values for the volatilome and for the microbiota were that obtained sampling just after adaptation of the microbiota to the bioreactor condition (Nissen et al., 2021a). Normalization of datasets was performed with the mean centering method.

6.3. Results and discussion

6.3.1. Volatilome analysis through SPME GC/MS

Through SPME GC-MS, among 30 duplicated cases (n = 60), 108 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA) and 69 significant VOCs were picked (ANOVA $p < 0.05$). The sintax for the name of molecules adopted in the present work is that of NIST database (NIST, USA), that are reported with initial capital letters (e.g. 1H-Indole, 3-methyl), while synonyms are reported with initial capital lowercase letter (e.g. skatole) (Casciano et al., 2021). 56 were relatively quantified at the baseline, while 69 were quantified during the 24 h of experiments at different timepoints. The 69 significant VOCS were then sorted and super-normalized for respective chemical identity, i.e., organic acids, aldehydes, ketones, alcohols, and aromatics (alkenes and amines). Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing different chemical classes (Nissen et al., 2020).

A PCA of 11 statistically significant organic acids distributed cases on the plot, separating the BL from time points of fermentation of the substrates, but not from those of the BC, and discriminating the controls CNO_2 and CO from the alternative formulations SA and SMA (Figure 1A). From our results, the main descriptors of fermentation with SA and SMA was Cyanic Acid methyl (by MANOVA approximately 42% and 49% of production, respectively) (Table S3). The descriptor of CO was principally Oxalic acid (approx. 98% of production) while those of CNO_2 were mainly Pentanoic and Hexanoic acids (approx. 58% and 67%, respectively). The contribution on short chain organic acids was not discriminated depending on the matrix (except for butanoic acids produced for the 45% by CNO_2), but it was on a time dependence (Table S4). Another interesting feature is that

the branched chain organic acids were all pushing to the quadrant relative to CNO_2, reaching high contribution ratio, as that of Pentanoic acid, 3-methyl, accounting for the 67% and totally produced at the EP (Table S3). Pentanoic acid, 3-methyl that is also known as a mucosal pro-inflammatory agent derived from protein fermentation (Wang, et al., 2020). From our results this is another feature of quality and safety that characterize the alternative formulations.

A PCA of 19 statistically significant alcohols distributed cases on the plot, separating the BL from time points of fermentation of the substrates, but not from those of the BC, and discriminating the controls CNO_2 and CO from each other and from the alternative formulations SA and SMA (Figure 1B). The descriptors of the controls were 1-Pentanol and 1-Hexanol for CNO_2 (both around the 36% of contribution in production) and Phenylethyl alcohol, Indole, and 1H-Indole, 3-methyl for CO (around 70%, 44%, and 92%, respectively). 1-Pentanol has antioxidant and prebiotic potential (Taneyo-Saa et al., 2014) also linked to *Akkermansia muciniphila* in healthy volunteers (Vernocchi et al., 2020). 1-Pentanol were similarly produced either by CNO_2 or SMA. This characteristic tells that SMA could compete with CNO_2, because is capable to produce positive alcohols and less detrimental alcohols that the control with no nitrite. The descriptors of the alternative formulations were 1-Heptanol for SMA (around 40%) and Cyclohexanol, 3,3,5-trimethyl-, acetate, cis- for SA (around 32 %) (Table S3). This last compound was produced mainly at the BL (Table S4), but after fermentation was uniquely addressed to SA, maybe re-arranged by the high amount of Acetate produced by SA.

A PCA of 15 statistically significant aldehydes distributed cases on the plot, separating the BL from time points of fermentation of the controls, but not completely from the time points of fermentation of alternative formulations. Also, CNO_2 and CO were discriminated from each other and from the alternative formulations SA and SMA (Figure 1C). SA and SMA did not have any specific descriptor. The aldehydes that described CNO_2 were Hexanal, Heptanal, and 2,4-Heptadienal, (E, E)- (53%, 42%, and 100% of contribution to total production, respectively) (Table S3) produced during fermentation (67 %, 81% and 100%) (Table S4). Those that described CO were Octanal, 2-Octenal, and Nonanal (48%, 42%, and 47%, respectively) (Table S3), although partially present at the BL (68%, 42%, and 46%) (Table S4). The profile generated by multivariate analysis of aldehydes described that the alternative formulations were not discriminated by typical oxidative aldehydes, that were instead specific descriptors of the controls. In particular, CNO_2 marked a unique signature of 2,4-Heptadienal, (E,E)- and along with CO was described by at least other six oxidative aldehydes, such as Hexanal, Octanal, Nonanal, 2-Nonenal,(E)-, 2-Octenal,(E)-. All these aldehydes are derived

from lipid oxidation of food and in a recent paper the effect of vegetal extract was efficaciously tested to be deterrent of their formation when added to roasted food in a similar *in vitro* model (Hu et al., 2022).

A PCA of 11 statistically significant ketones distributed cases on the plot, separating the BL from time points of fermentation of any substrates, but not of the BC. Also, CNO_2 and CO were discriminated from each other, but CNO_2 was partially separated from the alternative formulations. Moreover, SA and SMA did not discriminate much one to each other but had their specific descriptor (Figure 1D). CNO_2 did not have any specific descriptor. The ketones that described CO were 2-Hexanone and Cyclohexanone (around 87% and 56% of contribution on total production) ($p < 0.05$) (Table S3); the former was produced at the EP (100%) ($p > 0.05$), but the latter was also ascribed to be already present at the BL (around 43%) ($p < 0.05$) (Table S4). The descriptors of the alternative formulations were instead Acetylcyclopentanone for SMA (around 67%) ($p < 0.05$) and Cyclohexanone, 5-methyl-2-(1 -methylethyl)-, cis- for SA (around 39%) (Table S3), that were both absent at the BL and whose production was spread over the process either at T1 or EP ($p < 0.05$) (Table S4). More than products of oxidation, these two VOCs seemed linked to fermentation process. These two VOC were also unique signature of fermentation, because were absent at its beginning and were then produced homogeneously at the different time points of the process. Interestingly, Acetylcyclopentanone has been reported to provide protection in cell culture models from oxidative stress-induced toxicity and at a dose ranging from 0.80 to 2.40 nmol/kg of being able to prevent lethality in acetaminophen hepatotoxicity mouse model (Zhang et al., 2013).

A PCA of 13 statistically significant aromatic VOCs not previously sorted (accounting mainly for amines and alkenes) distributed cases on the plot, separating the BL from time points of fermentation of any substrates, but not of the BC. Also, CNO_2 and CO were discriminated from each other, but CNO_2 was partially separated from the alternative formulations. Lastly, SA and SMA did not discriminate much one to each other, and only SMA had a specific descriptor (Figure 1E). The VOCs that defined the controls were Cyanamide dibutyl for CNO_2 (around 48.9%) and Pyridine, 2,4,6-trimethyl for CO (around 94%) ($p < 0.05$) (Table S3). The former was absent at BL and was produced for the most at T1 (around 74%) while the latter accounted to contribute for around 12% at BL and around 84% at EP (Table S4). Interestingly, even considering chemical bias due to the high volatility of D-Limonene, this bioactive was a unique descriptor of SMA fermentation. In this case, D-Limonene enrichment was achieved during the whole process (around 33% and 49% at T1 and EP, respectively) starting from an initial amount (around 18%) ($p < 0.05$) (Table S4). D-Limonene

enrichment after SA fermentation was maybe due to increased biodisponibility for the progression of fermentation that should liberate this VOC surely sourced from the vegetal extract of SMA. The positive impact that this VOC could generate on the host mucosa have been extensively studied and also its retainment and enrichment during colonic fermentation within in vitro model has been similarly assessed in the past. Another remarkable attribute to address to the alternative formulations is the lack of the negative impact that could bring the exposure to Cyanamide dibutyl, that was instead describing the control with nitrite. In fact, nitrites have the capacity to form N-nitrous carcinogenic compounds, as in the human colon there are amines and amides derived from the bacterial metabolism of aminoacids, that could be N-nitrous in presence of heme nitrosylate derived from not absorbed residual of red meat (Herrmann et al., 2015; Johnson, 2017; Meurillon e Engel, 2016).

CASE STUDY 3: Alternative formulations of salami

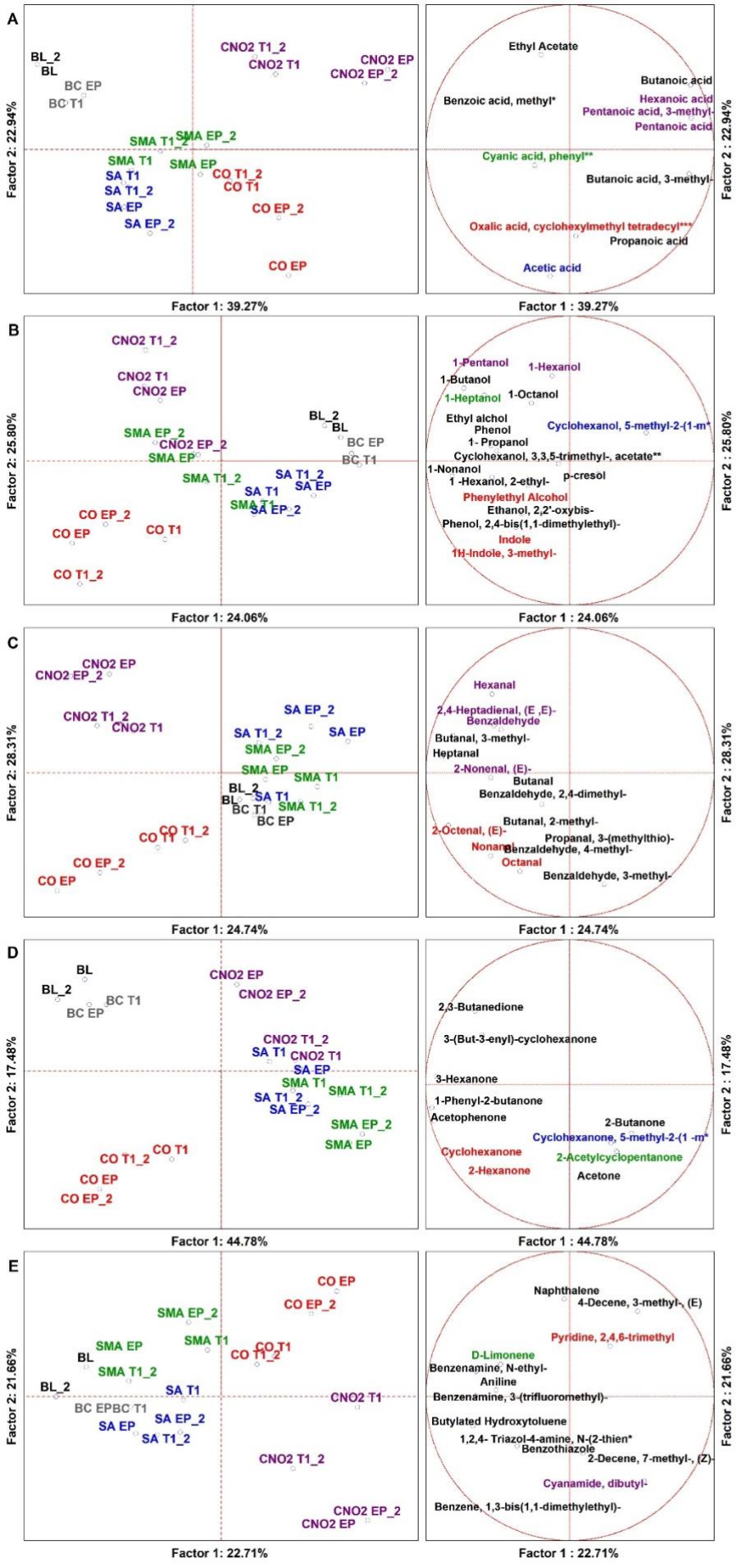

Figure 1. PCAs of the volatilome sorted by chemical classes of significant (ANOVA $p < 0.05$) VOCs, including the biological replicas of SA, SMA, CNO2, CO, BC, and the baseline (BL) and different time points (T1 = 18 h and EP = 24 h). **A**) Acids; G) Alcohols; **C**) Aldehydes; **D**) Ketones; **E**) Other aromatic VOCs. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of the respective group of cases. SA = Salami with ascorbate; SMA = Salami with ascorbate and herbal extract; CO = control with no nitrate; CNO_2 = commercial control with nitrate.

6.3.2. Shift of main microbial metabolites

6.3.2.1. Beneficial postbiotic VOCs

Three short chain and two medium chain organic acids were considered, namely Acetic, Propanoic, Butanoic, Pentanoic, and Hexanoic acids. The absolute quantifications at the baseline (Table S3) were compared to that at the two time points, T1 and EP, and the difference measured and normalized (Figure 2). Considering, the shift of fermentations in comparison to the BL, from the recipient analyses the results demonstrated that any type of fermentations tested was able to produce low molecular organic acids. In particular, the best fermentation outputs were generated by the control sample with nitrites (CNO2). Among the alternative formulation, SA fermentation was able to produce almost 7 times more acetic and 4 times more either Pentanoic or Hexanoic acids, than the BL, while fermentation of SMA produced few amounts of the five VOCs. So far, the trend in beneficial postbiotic VOCs production was CNO2 > SA > SMA > CO.

Considering the production of organic acids, the alternative formulations were able to compete with the commercial products for the production of short chain fatty acids, but not for that of medium chain fatty acids. Eventually, the formulation SA generated more organic acids of any kind in respect to SMA. Other authors have found that the addition of herbal extract and fibers to sausages can produce an higher amount of SCFA in respect to a commercial control, but no influence was ascribed to the presence or not of nitrates (Perez-Burillo et al., 2019).

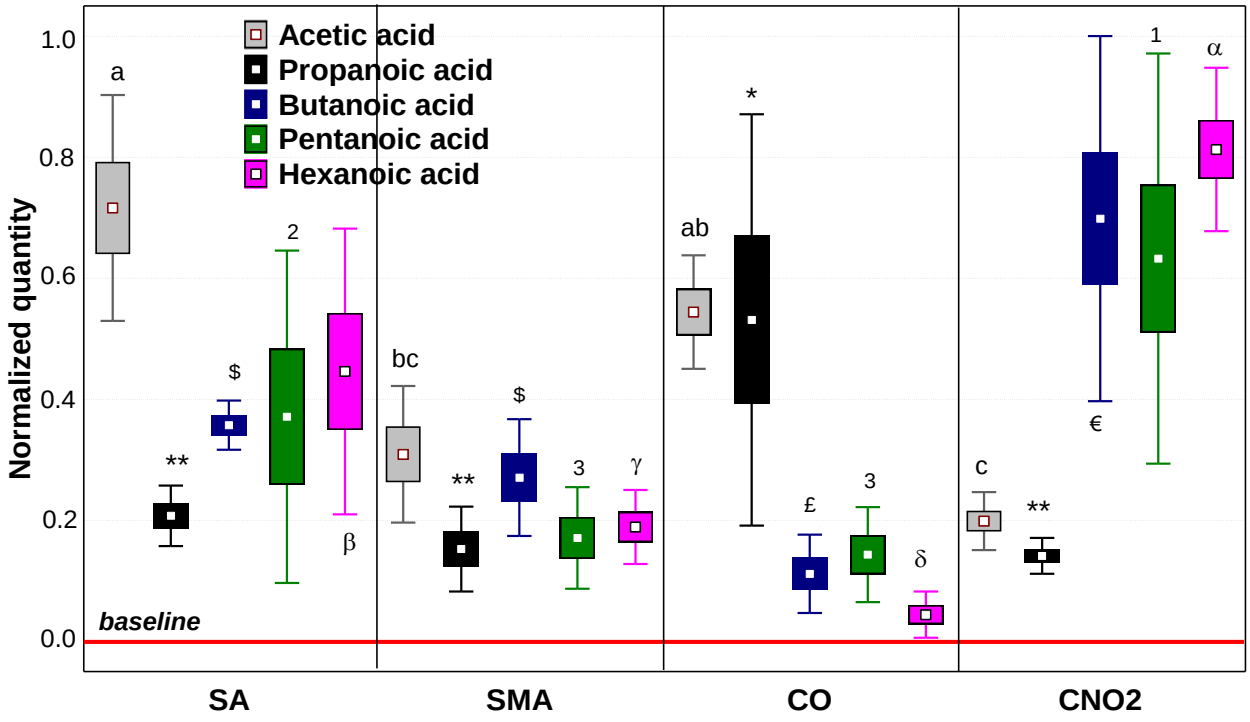

Figure 2. Changes in the abundance of beneficial microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S3). Changes were recorded after 18, and 24 h of *in vitro* fecal batch fermentations with SA, SMA, and controls CO and CNO2. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments ($n = 4$). Marker = mean; box = mean ± S.D.; whiskers = Confidence Interval 0.95. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to ANOVA model followed by Tukey's HSD test ($p < 0.05$). SA = Salami with ascorbate; SMA = Salami with ascorbate and herbal extract; CO = control with no nitrite; CNO_2 = commercial control with nitrate.

6.3.2.2. Detrimental VOCs

Fermentation of any kind of salami has produced VOCs that are potentially detrimental and toxic for the host mucosa, that are derived from lipid oxidation and aminoacids (Tyrosine, Triptophan, Phenilanaline) fermentation. In particular, Phenol, Phenol, 2-methyl (a.k.a. p-Cresol), Indole, and 1H-Indole. 3-methyl (a.k.a. Skatole). Starting from physiological concentrations of these VOCs at the baseline (Table S4), the sample that generated the highest amount was the control with no nitrite (CO) (Figure 3). In respect to this control, SA, SMA and CNO_2 produced similar overall amounts of any compounds ($p > 0.05$), except Indole ($p < 0.05$) and Skatole (not detected in SA), approximately 6 times less than CO ($p < 0.05$). The trend of production of these VOCs was: CO > CNO_2 > SMA > SA. Skatole is a toxic product of the bacterial decarboxylation of tryptophan by *Bacteroides* spp. and *Clostridium* spp., which affect the mucosa and causes the production of inflammatory cytokines (Roager and Licht, 2018). Phenol and p-cresol are shown to impair epithelial barrier function in vitro

and may be targeted for carcinogens, (Wang et al., 2020). P-cresol and Indole, for example, would be transformed into p-Cresyl sulphate and Indoxyl sulphate which after conjugation accumulates in the liver leading to complications and pathologies such as chronic kidney diseases and cardiovascular diseases (Wu et al., 2011; Arcidiacono et al., 2022).

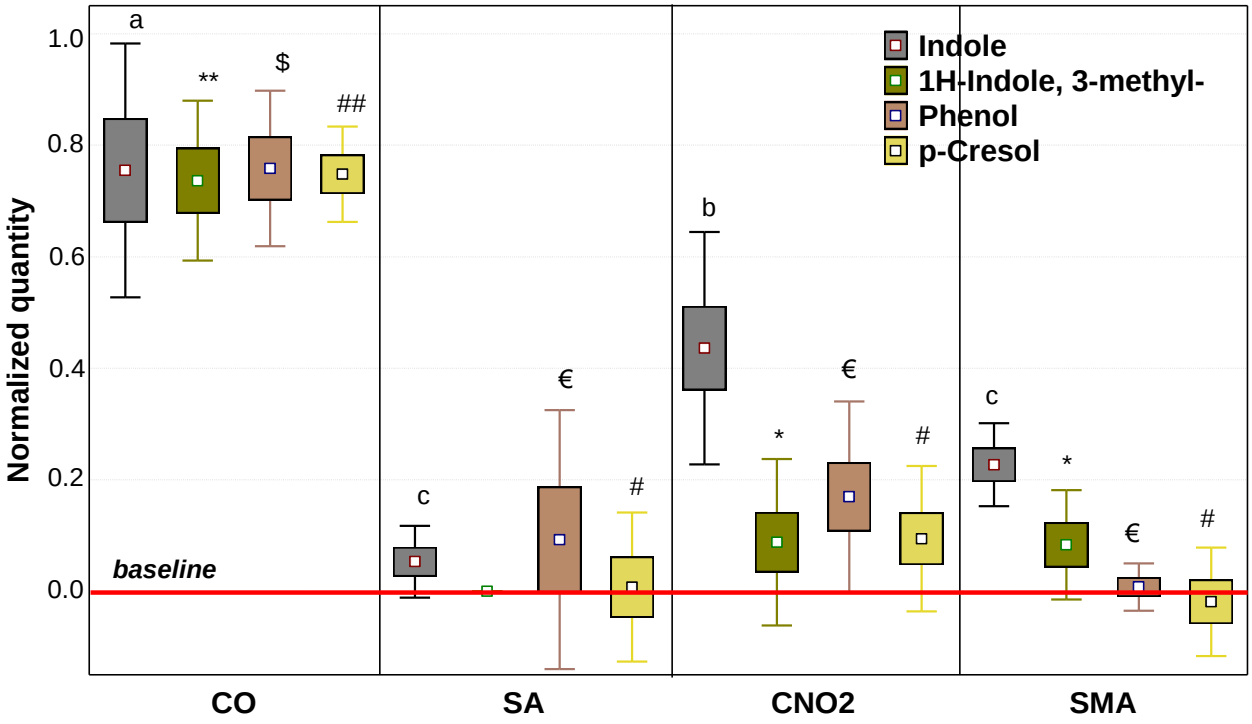

Figure 3. Changes in the abundance of detrimental microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S4). Changes were recorded after 18, and 24 h of *in vitro* fecal batch fermentations with SA, SMA, CO, and CNO_2. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments (n = 4). Marker = mean; box = mean ± S.D.; whiskers = Confidence Interval 0.95. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to ANOVA model followed by Tukey's HSD test ($p < 0.05$). SA = Salami with ascorbate; SMA = Salami with ascorbate and herbal extract; CO = control with no nitrite; CNO_2 = commercial control with nitrate.

6.3.3. Microbiota analyses of colonic fermentations

qPCR absolute quantifications were targeted to 16 different bacterial taxa related to the core microbiota of the human colon, including total Eubacteria, *Bacteroidetes* and *Firmicutes* to describe the large picture; *Lactobacillales*, *Bifidobacteriaceae, Clostridium* group IV, *Bifidobacterium longum, Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Faecalibacterium prausnitzii*, and

Akkermansia muciniphila to describe the commensal beneficial part of the core colon microbiota; and *Enterobacteriaceae*, *Clostridium* group I, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Escherichia coli* (total), *Escherichia coli* (toxigenic), *Desulfovibrio*. spp., to describe the commensal opportunistic part of the core colon microbiota (Table S5).

6.3.3.1. Shift in taxa relative to the core microbiota

Considering the total Eubacteria (Table 2), in respect to the abundances at the BL and apart from the values of the blank control (BC) (Table 2), the alternative formulations SA and SMA at EP fostered significantly the growth of Eubacteria ($p < 0.05$), alike the commercial control CNO_2, and almost thrice than the negative control CO.

The quantifications of *Bacteroidetes* phylum (Table 2) have shown changes principally at EP, when any samples, but SA, had significant differences in respect to BL ($p < 0,05$). In particular, fermentations of CO and CNO_2 triggered a reduction, while that of salami fostered a growth. SMA was the best performer, able to increase at the EP the loads of this taxon significantly up to 1.25E+10 ± 4.94E+9 cells/mL ($p < 0.05$), that was 1.7 and 4 times more than the quantity recorded by SA and by the commercial control CNO_2, respectively. *Bacteroidetes* showed increases just after fermentation of the alternative formulations, expressing a positive feature, since many species between this main phylum are important commensal and fibrolytic specialist. Also in this view, the higher increase in *Bacteroidetes* taxon scored by SMA in respect to SA, could be due to the higher presence of vegetal fiber brought by the vegetal antioxidant extract included in this formulation. Other authors have reported that the addition of a fiber supplement in sausages, once fermented in a similar *in vitro* model increased the quantity of *Bacteroidetes* of comparable levels (Perez-Burillo et al., 2019).

Considering *Firmicutes* (Table 2), significant increases were observed at EP for any sample, but SMA ($p > 0.05$). Although, the surges after fermentation with both the controls were quite the double in respect to those of the alternative formulations. For example, at the end point CNO_2 had level of this taxon at 6.56 E+09 ± 1.71 E+09 cells/mL, that was 1.90 times higher than SA. The trends of the shift of *Firmicutes* observed concerning this taxon were telling of increases in any substrates, but this outcome has to be differently considered in the view of trends happened at lower taxonomic levels.

Based on the values of quantifications relative to *Firmicutes* and *Bacteroidetes*, the ratios F/B (Table 2) was calculated to consider changes in the condition of eubiosis defined at the BL. After colonic fermentation, SMA and SA were able to keep the ratio similar to the BL ($p > 0.05$), but the controls were not ($p > 0.05$). In particular, the ratio of CO was higher than 3, indicating a microbiota dysbiosis due to the overrepresentation of *Firmicutes*. So far, the intensity of the capacity to maintain the

microbiota eubiosis among the salami tested was: SMA > SA > CNO_2 > CO. *Firmicutes* e *Bacteroidetes* are the two principal bacterial phyla that live the adult human colon. The ratio of their abundances is an index of microbiota eubiosis and values higher than 2 are commonly associated with *in vivo* microbiota dysbiosis (Koliada et al., 2017; Zhou et al., 2017). In our samples, starting from an eubiosis condition relative to the well-being of the donors, SA and SMA were able to keep it up to the end point, in contrast with the results of the controls. Other authors showed that the addition of fiber to sausages can increase the abundance of *Bacteroidetes* and reduce that of *Firmicutes*, eventually repealing unbalances in F/B (Perez-Burillo et al., 2019).

Table 2. Quantification of Eubacteria, *Bacteroidetes* e *Firmicutes* and the *Firmicutes* to *Bacteroidetes* ratio.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Eubacteria	**BL**	**T1**	**EP**	
CO	2.77E+10 ± 8.47E+09	0.02	0.58^{AB}	*0.810581*
SA	2.77E+10 ± 8.47E+09^{b}	0.75^{ab}	1.63^{aA}	*0.023662*
SMA	2.77E+10 ± 8.47E+09^{b}	0.87^{ab}	1.38^{aA}	*0.009666*
CNO_2	2.77E+10 ± 8.47E+09^{b}	0.94^{a}	1.44^{aA}	*0.019003*
BC	2.77E+10 ± 8.47E+09^{a}	-0.15^{a}	-1.74^{bB}	*0.015644*
		0.107124	*0.000950*	***p* value**
Firmicutes	**BL**	**T1**	**EP**	
CO	2.46E+09 ± 2.08E+08^{b}	1.43^{aA}	1.44^{aA}	*0.000003*
SA	2.46E+09 ± 2.08E+08	-0.02^{B}	0.49^{B}	*0.064013*
SMA	2.46E+09 ± 2.08E+08	0.50^{AB}	0.71^{AB}	*0.098002*
CNO_2	2.46E+09 ± 2.08E+08^{b}	-0.39^{bB}	1.42^{aA}	*0.000457*
BC	2.46E+09 ± 2.08E+08^{b}	0.91^{abAB}	1.28^{aA}	*0.000134*
		0.000012	*0.002652*	***p* value**
Bacteroidetes	**BL**	**T1**	**EP**	
CO	4.80E+09 ± 1.84E+09^{a}	-0.22^{ab}	-1.16^{bB}	*0.049707*
SA	4.80E+09 ± 1.84E+09	0.15	0.59^{A}	*0.070006*
SMA	4.80E+09 ± 1.84E+09^{b}	0.11^{b}	1.38^{aA}	*0.005644*
CNO_2	4.80E+09 ± 1.84E+09^{a}	-0.11^{ab}	-0.62^{bB}	*0.000008*
BC	4.80E+09 ± 1.84E+09^{a}	0.11^{a}	-2.62^{bC}	*0.006603*
		0.080616	*0.000444*	***p* value**
F/B	**BL**	**T1**	**EP**	
CO	0.51 ± 0.22^{c}	1.61 ± 0.36^{bA}	3.10 ± 0.61^{aB}	*0.000003*
SA	0.51 ± 0.22	0.46 ± 0.10^{B}	0.48 ± 0.18^{C}	*0.160113*
SMA	0.51 ± 0.22	0.67 ± 0.14^{B}	0.32 ± 0.10^{C}	*0.076002*
CNO_2	0.51 ± 0.22^{b}	0.42 ± 0.17^{bB}	2.10 ± 0.67^{aB}	*0.000457*
BC	0.51 ± 0.22^{b}	0.95 ± 0.29^{bA}	7.64 ± 0.86^{aA}	*0.000134*
		0.000011	*0.002652*	***p* value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA P value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SA = Salami with ascorbate; SMA = Salami with ascorbate and herbal extract; CO = control with no

nitrite; CNO_2 = commercial control with nitrate; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation. F/B = *Firmicutes* to *Bacteroidetes* ratio.

6.3.3.2. Commensals and beneficial taxa

Stating generally that a salami substrate can foster partially beneficial microbes, from our results we have indeed found increased abundances of major taxa at the class or family level, but then going deeper to genus or species levels we just have found reductions in abundances. Thus, here the results will be discussed comparing weather is the sample that limited more the loss of beneficial taxa.

Amongst the beneficial bacteria that were targeted, *Lactobacillales* and *Bifidobacteriaceae* had a different trend during fermentation (Table 3). At the EP in respect to the BL, the former taxon increased after fermentation with any substrate, but significantly just for SA ($p < 0.05$), while the latter increases significantly just for SA and SMA and decreased significantly for CNO_2 ($p < 0.05$). After SA fermentation, at the EP *Lactobacillales* loads was 2.10E+07 ± 3.92E+06 cells/mL, more than the double of that of CNO_2. At the higher levels *Lactobacillales* were fostered by any samples, but the alternative formulations were better. This is a feature previously observed and could be due to the contribute of the starter consortium and the indigenous species present in salami, of which many are part of *Lactobacillales* (Pini et al., 2020).

Also the *Bifidobacteriaceae* were increased just by the alternative formulations. After SMA fermentations, at the EP *Bifidobacteriaceae* accounted for 8.63E+08 ± 2.97E+08 cells/mL, 7.3 times more than CNO_2. Within this family, *B. longum* was particularly affected by the controls recording dramatic losses after their fermentations but surged significantly ($p < 0.05$) and similarly ($p > 0.05$) in abundances after SA and SMA fermentations. In a recent work, the *in vitro* fermentation of salami including inulin promoted *Bacteroides* and *Bifidobacterium* (Perez-Burillo et al., 2019).

Among *Clostridiales*, the *Clostridium* group IV and the recipient *Faecalibacterium prausnitzii* were underrepresented after any fermentation, but SMA, that anyhow scored no shift in respect to the BL ($p > 0.05$). The group BPP, that mainly targets the *Bacteroides* genus, increased significantly at the EP just for SMA ($p < 0.05$), as we previously have observed at the phylum level. Lastly, *Akkermansia muciniphila* was not fostered by any substates.

Table 3. Quantification of commensal benefical taxa.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Lactobacillales	**BL**	**T1**	**EP**	
CO	4.80E+06 ± 3.64E+05	0.56	0.82^{AB}	*0.681632*
SA	$4.80E+06 ± 3.64E+05^{b}$	0.72^{ab}	2.13^{aA}	*0.001842*
SMA	4.80E+06 ± 3.64E+05	0.94	1.74^{A}	*0.165766*
CNO_2	4.80E+06 ± 3.64E+05	0.72	1.10^{AB}	*0.418668*

BC	4.80E+06 ± 3.64E+05^{a}	0.44^{ab}	-1.34^{bB}	*0.048217*
		0.932608	*0.043956*	***p* value**
Bifidobacteriaceae	**BL**	**T1**	**EP**	
CO	4.10E+08 ± 4.77E+07	-0.11^{B}	0.48^{AB}	*0.056606*
SA	4.10E+08 ± 4.77E+07^{b}	0.08^{abAB}	1.04^{aA}	*0.003197*
SMA	4.10E+08 ± 4.77E+07^{c}	1.01^{bA}	2.04^{aA}	*0.013858*
CNO_2	4.10E+08 ± 4.77E+07	-0.52^{B}	-0.83^{B}	*0.058814*
BC	4.10E+08 ± 4.77E+07^{a}	-0.32^{B}	-2.43^{C}	*0.046692*
		0.0003459	*0.000315*	***p* value**
***Clostridium* Group IV**	**BL**	**T1**	**EP**	
CO	1.36E+08 ± 1.83E+07^{a}	-1.44^{bB}	-1.38^{bB}	*0.000121*
SA	1.36E+08 ± 1.83E+07	0.11^{A}	-0.46^{bAB}	*0.054040*
SMA	1.36E+08 ± 1.83E+07	0.02^{A}	-0.02^{A}	*0.902609*
CNO_2	1.36E+08 ± 1.83E+07^{a}	-0.64^{bAB}	-1.19^{bB}	*0.000436*
BC	1.36E+08 ± 1.83E+07^{a}	0.28^{aA}	-1.81^{bB}	*0.000024*
		0.000021	*0.000011*	***p* value**
B. longum	**BL**	**T1**	**EP**	
CO	1.08E+08 ± 1.52E+07^{a}	-1.70^{bB}	-1.41^{bB}	*0.043768*
SA	1.08E+08 ± 1.52E+07	0.03^{A}	0.96^{A}	*0.023466*
SMA	1.08E+08 ± 1.52E+07^{b}	0.44^{bA}	1.37^{aA}	*0.000166*
CNO_2	1.08E+08 ± 1.52E+07	-3.63^{C}	-3.86^{C}	*0.000001*
BC	1.08E+08 ± 1.52E+07	-2.02^{bBC}	-3.38^{cC}	*0.000003*
		0.000429	*0.000001*	***p* value**
A. muciniphila	**BL**	**T1**	**EP**	
CO	4.03E+05 ± 7.74E+04	-0.15	-0.07^{A}	*0.063285*
SA	4.03E+05 ± 7.74E+04^{a}	-1.19^{b}	-1.52^{bB}	*0.000062*
SMA	4.03E+05 ± 7.74E+04^{a}	-0.91^{ab}	-1.08^{bAB}	*0.000034*
CNO2	4.03E+05 ± 7.74E+04	-0.27	-0.79^{AB}	*0.055117*
BC	4.03E+05 ± 7.74E+04^{a}	-0.51^{a}	-3.06^{bC}	*0.000004*
		0.1051502	*0.000001*	***p* value**
F. prausnitzii	**BL**	**T1**	**EP**	
CO	1.26E+04 ± 3.18E+03^{a}	-0.66^{b}	-1.41^{bA}	*0.001359*
SA	1.26E+04 ± 3.18E+03^{a}	0.42^{a}	-0.77^{bAB}	*0.061564*
SMA	1.26E+04 ± 3.18E+03	0.05	-0.41^{A}	*0.191359*
CNO_2	1.26E+04 ± 3.18E+03	-0.15	-0.83^{AB}	*0.072828*
BC	1.26E+04 ± 3.18E+03^{a}	0.28^{a}	-1.82^{bB}	*0.001090*
		0.090691	*0.023811*	***p* value**
BPP group	**BL**	**T1**	**EP**	
CO	6.82E+09 ± 3.05E+08	-0.27	-0.29^{A}	*0.918863*
SA	6.82E+09 ± 3.05E+08	-0.13	-0.47^{B}	*0.512972*
SMA	6.82E+09 ± 3.05E+08^{b}	0.03^{b}	1.16^{aA}	*0.005472*
CNO_2	6.82E+09 ± 3.05E+08^{a}	-0.68^{a}	-2.23^{b}	*0.025051*
BC	6.82E+09 ± 3.05E+08^{a}	-1.70^{b}	-2.54^{bC}	*0.009275*
		0.244794	*0.000006*	***p* value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SA = Salami with ascorbate; SMA = Salami with ascorbate and herbal extract; CO = control with no nitrite; CNO_2 = commercial control with nitrate; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

6.3.3.3. Commensals opportunistic taxa

To evaluate the shifts during colonic fermentation of a portion of the opportunistic part of the microbiota, we have selected specìfic taxa that have strong proteolysis activity and are also associated with western diet enterotype, namely *Enterobacteriaceae*, *Clostridium* group I, *Atopobium* - *Collinsella* - *Eggerthella* (ATOP) group, *Escherichia coli* and *Desulfovibrio* spp. (Table 4).

Considering that by our in vitro model it was not possible to evidence many reductions in bacterial taxa due to the nature of the fermentative substrates employed, that was rich in protein and rich in lipid of animal origin, but it was possible to quantify and discuss the limited growth of these taxa in comparison to the controls.

From our results, SA and SMA always limited more the growth of opportunistic in comparison to the controls. SA was more potent than SMA, because made these taxa grew less in four out of six cases. Any substrate tested was able to foster *Enterobacteriaceae* from the BL to EP, although SA did not signficantly ($p > 0.05$). The same trend was observed within this family, in fact the substrates fermentations made total *E. coli* to increase. For both these taxa, the increment at EP was anyhow minor in SA and SMA than in the controls and differently significant when compared to CO ($p < 0.05$). From the total population of *E. coli* (5.04E+05 ± 2.08E+04 cells/mL) we have found a small portion (2.94E+02 ± 5.10E+01 cells/mL) that harboured the Cytolethal Distending Toxin and could potentially become pathogenic. Interestingly, this taxon was reduced by the alternative salami and fostered by the controls, although both not significantly ($p > 0.05$). In particular, the top reduction was obtained after fermentations of SMA. In contrast to our findings, in a recent paper done with similar methodologies, but different in vitro model, the authors have found that adding citrus fibers to salami also reduced the prevalence of *Escherichia/ Shigella* group.

The fermentation of SA made the ATOP group grow less than that of CO ($p < 0.05$). Any fermented substrate made *Clostridum* group I grow significantly, with SMA as the sample that made this group grow less, but the results were similar among the samples ($p > 0.05$). Lastly, significant shifts were observed also for the genus *Desulfovibrio* relative to increase due to the CNO_2 and decrease due to SA, with the control accounting for a load at the EP that was 7.4 times higher than that of the alternative salami. *Desulfovibrio* is a sulfurate-reducer genus able to affect and shrink the mucin barrier and therefore the integral structure of the colon mucosa by production of Dimethyl sulfate and also inducer of colitis (Rowan et al., 2010). This reduction of this taxon with SA is evidence that the absence of nitrite in formulation, which uses to be a substrate for this harmful taxon (Warren, Citron, Merriam, Goldstein, 2005), results in its containment, and also in its reduction due principally to the inhibitory action of ascorbate.

Lastly, as seen from relative values of quantifications shown in previous tables we discuss the results of the ecological competition between *Enterobacteriaceae* and *Bifidobacteriaceae*. This is an index of bifidogenic capacity of the substates unveiling possible prebiotic features. The competition had shown to be higher when the alternative formulations were fermented in respect to the controls. In this context, SMA was more potent than SA; maybe because, even if ascorbate of SA has the capacity to limit more *Enterobacteriaceae* than SMA, the vegetal extract of SMA induced a higher growth of *Bifidobacteriaceae*, as it is reported that vegetal fibers use to foster this health-related family (Wang et al., 2019).

Table 4. Quantification of commensal opportunistic taxa.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Enterobacteriaceae	**BL**	**T1**	**EP**	
CO	7.85E+07 ± 4.58E+05^{b}	0.84^{abB}	2.56^{aA}	*0.049707*
SA	7.85E+07 ± 4.58E+05	0.56^{B}	0.70^{B}	*0.052006*
SMA	7.85E+07 ± 4.58E+05^{b}	0.94^{abB}	1.21^{aB}	*0.005644*
CNO_2	7.85E+07 ± 4.58E+05^{b}	1.09^{aB}	1.86^{aB}	*0.000008*
BC	7.85E+07 ± 4.58E+05^{c}	2.32^{bA}	3.93^{aA}	*0.006603*
		0.000616	*0.000444*	***p* value**
Gruppo ATOP	**BL**	**T1**	**EP**	
CO	5.29E+05 ± 1.09E+05^{b}	0.16^{b}	1.21^{aA}	*0.024434*
SA	5.29E+05 ± 1.09E+05	0.08	0.21^{B}	*0.880294*
SMA	5.29E+05 ± 1.09E+05	0.27	0.40^{AB}	*0.574153*
CNO_2	5.29E+05 ± 1.09E+05^{b}	0.72^{ab}	1.07^{aAB}	*0.049402*
BC	5.29E+05 ± 1.09E+05^{b}	0.28^{b}	1.91^{aA}	*0.042082*
		0.852626	*0.026102*	***p* value**
***Clostridium* gruppo I**	**BL**	**T1**	**EP**	
CO	1.54E+04 ± 3.06E+03^{b}	1.68^{a}	2.49^{aAB}	*0.000208*
SA	1.54E+04 ± 3.06E+03^{b}	1.24^{a}	2.48^{aAB}	*0.000308*
SMA	1.54E+04 ± 3.06E+03	0.93	1.27^{B}	*0.245233*
CNO_2	1.54E+04 ± 3.06E+03^{b}	1.80^{a}	2.03^{aAB}	*0.022968*
BC	1.54E+04 ± 3.06E+03^{b}	1.67^{a}	3.05^{aA}	*0.000087*
		0.453844	*0.018590*	***p* value**
Escherichia coli* (total)	**BL**	**T1**	**EP**	
CO	5.04E+05 ± 2.08E+04^{b}	0.62^{b}	2.28^{aA}	*0.031012*
SA	5.04E+05 ± 2.08E+04	0.52	0.69^{B}	*0.072121*
SMA	5.04E+05 ± 2.08E+04	0.74	1.03^{B}	*0.080023*
CNO_2	5.04E+05 ± 2.08E+04^{b}	0.89^{a}	1.36^{aB}	*0.034346*
BC	5.04E+05 ± 2.08E+04^{b}	1.89^{a}	3.79^{aA}	*0.000019*
		0.082102	*0.035284*	***p* value**
E. coli* (potentially toxigenic)*				
CO	2.94E+02 ± 5.10E+01	0.55	1.45	*0.340221*
SA	2.94E+02 ± 5.10E+01	0.20	-0.17	*0.941371*
SMA	2.94E+02 ± 5.10E+01	-0.43	-1.01	*0.698634*
CNO_2	2.94E+02 ± 5.10E+01	1.08	1.09	*0.674387*
BC	2.94E+02 ± 5.10E+01	0.85	0.29	*0.490869*

		0.745796	*0.250029*	***p* value**
***Desulfovibrio* spp.**				
CO	1.35E+06 ± 1.59E+05	1.33^{A}	1.25^{A}	*0.090244*
SA	1.35E+06 ± 1.59E+05^{b}	-0.12^{bC}	-1.12^{bC}	*0.000012*
SMA	1.35E+06 ± 1.59E+05	0.19^{B}	0.21^{B}	*0.065024*
CNO_2	1.35E+06 ± 1.59E+05^{b}	$0.90^{a\,A}$	$1.77^{a\,A}$	*0.000001*
BC	1.35E+06 ± 1.59E+05	0.13^{B}	0.65^{B}	*0.080410*
		0.000012	*0.000005*	***p* value**

*This taxon was amplified by targeting cell division protein (FtsZ) rDNA; **This taxon was amplified by targeting Cytolethal Distending Toxin rDNA; Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA P value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SA = Salami with ascorbate; SMA = Salami with ascorbate and herbal extract; CO = control with no nitrite; CNO_2 = commercial control with nitrate; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

6.4. Conclusions

The onset of pathologies in the gastrointestinal tract due to the excessive consumption of red meat has recently prompted the food industries to seek alternative strategies. In particular, the processed meat industry is studying alternative formulations in the production of salami. One of the main strategies is that aimed at replacing nitrites, which in the host can lead to the formation of toxic compounds (e.g. nitrosamines).

In the following study, innovative formulations were evaluated in which the nitrites were replaced by ascorbic acid and / or a mix of plant antioxidants.

The results obtained show that the innovative formulations promote a general eubiosis of the intestinal microbiota, in the face of those preselected indices including favorable F/B ratio, proliferation of beneficial microbial taxa including *Lactobacillales, Bifidobacteriaceae* and reduction of negative microbial populations, including *Enterobacteriaceae* and ATOP group. Furthermore, the volatiloma analysis highlights a marked production of beneficial molecules, including short-chain fatty acids such as Acetate, Propionate and Butyrate, and a reduction in host negative molecules such as Phenol and p-Cresol, resulting from the fermentation of proteins. Although the innovative formulations have not given benefits clearly superior to those of the control and the product with nitrites, the results obtained are promising, as the antioxidants used in place have given results comparable to those obtained with the traditional formulation.

These results may represent an encouraging starting point for the processed meat industry for the development of innovative formulations aimed at reducing the negative impact of these products on consumer health.

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6.6. Supplementary materials

Table S1. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors significant among the food matrices.

VOCs	**Matrix**						***p value***
	Baseline	**CO**	**SA**	**CNO2**	**SMA**	**BC**	
Acetic acid	2.13	36.46	26.39	8.23	15.02	11.76	0.213495
Propanoic acid	3.53	31.07	17.68	22.28	12.97	12.45	0.658082
Butanoic acid	4.21^{b}	6.91^{b}	17.21^{b}	44.68^{a}	17.78^{b}	9.18^{b}	0.000206
Pentanoic acid	3.45^{b}	12.72^{b}	19.64^{ab}	42.54^{a}	11.76^{b}	9.87^{ab}	0.016767
Hexanoic acid	0.00^{b}	2.11^{b}	31.29^{a}	58.32^{a}	8.26^{b}	0.00^{b}	0.005048
Butanoic acid, 3-methyl-	3.12	25.12	11.29	34.58	25.86	0.00	0.332755
Pentanoic acid, 3-methyl-	0.00	10.28	0.00	67.01	15.55	7.14	0.195487
Ethyl Acetate	34.99^{a}	5.64^{b}	4.03^{b}	24.86^{a}	6.36^{b}	24.09^{a}	0.000230
Benzoic acid, methyl ester	48.51^{a}	12.04^{b}	10.53^{b}	9.13^{b}	10.18^{b}	9.58^{b}	0.000043
Cyanic acid, phenyl ester	8.72	0.00	42.08	0.00	49.19	0.00	0.160237
Oxalic acid, cyclohexylmethyl tetradecyl ester	0.00	98.51	1.48	0.00	0.00	0.00	0.097553
1-Hexanol	23.01	9.41	7.81	36.63	14.22	8.89	0.046744
1 -Hexanol, 2-ethyl-	22.44	26.86	6.00	21.22	23.46	0.00	0.117057
1- Propanol	10.73	20.46	14.36	27.78	19.77	6.88	0.365735
1-Butanol	13.31	20.15	13.94	23.43	25.87	3.27	0.297916
1-Heptanol	14.53	18.09	0.00	26.75	40.61	0.00	0.669204
1-Nonanol	0.00	45.21	7.81	27.53	19.43	0.00	0.025002
1-Octanol	19.87	21.11	15.65	25.33	9.46	8.54	0.574500
1-Pentanol	10.19	17.06	10.16	36.14	24.97	1.45	0.374614
Ethyl alchol	7.33	22.57	10.18	22.22	33.27	4.40	0.046411
Ethanol, 2,2'-oxybis-	11.61	37.53	13.12	7.59	4.84	25.27	0.277286
Cyclohexanol, 3,3,5-trimethyl-, ac**	11.47	19.65	19.27	15.07	17.08	17.43	0.867343

Cyclohexanol, 5-methyl-2-(1-m*	69.61^{a}	0.00^{b}	30.38^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.000839
Benzothiazole	13.66	23.19	21.82	10.19	21.26	9.85	0.018237
Phenol	5.18	29.21	5.89	21.39	35.42	2.87	0.502411
Phenol, 2,4-bis(1,1-dimethylethyl)-	6.59	19.46	16.82	15.95	29.19	11.96	0.117416
p-cresol	29.70	19.39	13.01	18.06	12.93	6.88	0.022734
Phenylethyl Alcohol	9.53	70.33	7.03	6.396	6.69	0.00	0.013259
Indole	14.70	44.72	1.71	7.29	29.43	2.13	0.045792
1H-Indole, 3-methyl-	0.00^{b}	92.31^{a}	0.00^{b}	0.00^{b}	7.689^{b}	0.00^{b}	<0.000001
Butanal	0.00^{b}	31.07^{a}	0.00^{b}	22.38^{a}	46.53^{a}	0.00^{b}	0.008196
Butanal, 2-methyl-	18.95	17.52	10.47	13.95	27.39	11.68	0.655223
Butanal, 3-methyl-	10.32^{ab}	19.12^{ab}	19.54^{ab}	46.65^{a}	2.87^{b}	1.47^{b}	0.010140
Propanal, 3-(methylthio)-	1.68	28.22	30.97	0.00	21.38	17.73	0.132395
2-Nonenal, (E)-	0.00^{b}	26.02^{a}	10.05^{ab}	24.62^{a}	10.83^{ab}	28.46^{a}	0.001634
2-Octenal, (E)-	24.59^{ab}	42.06^{a}	0.00^{b}	33.34^{a}	0.00^{b}	0.00^{b}	0.000020
Heptanal	16.47^{ab}	25.26^{a}	5.88^{b}	42.32^{a}	0.00^{b}	10.05^{b}	0.000363
Hexanal	5.03	6.95	14.64	53.68	16.83	2.85	0.119237
Nonanal	28.19^{ab}	46.92^{a}	1.21^{b}	16.22^{b}	7.43^{b}	0.00^{b}	0.000410
Octanal	48.89^{ab}	48.34^{a}	0.00^{b}	0.00^{b}	0.00^{b}	2.76^{ab}	0.011820
Benzaldehyde	9.54	17.91	18.01	24.51	19.80	10.21	0.174502
Benzaldehyde, 2,4-dimethyl-	30.10	17.75	13.90	18.41	19.83	0.00	0.210582
Benzaldehyde, 3-methyl-	14.18^{abc}	32.56^{ab}	18.71^{abc}	3.85^{c}	23.22^{ab}	7.46^{bc}	0.002231
Benzaldehyde, 4-ethyl-	18.94	18.85	16.31	15.62	16.27	13.99	0.986743
1-Phenyl-2-butanone	67.95^{a}	32.04^{ab}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.006868
2,3-Butanedione	75.66^{a}	0.00^{d}	2.96^{cd}	7.62^{bc}	1.20^{cd}	12.54^{bc}	<0.000001
2-Acetylcyclopentanone	0.00^{b}	8.17^{b}	13.10^{b}	11.32^{b}	67.39^{a}	0.00^{b}	0.000042
2-Butanone	14.06^{b}	17.92^{ab}	25.85^{a}	16.62^{b}	16.66^{b}	8.86^{b}	0.003377
2-Hexanone	0.00	86.92	0.00	0.00	13.07	0.00	0.146963
3-(But-3-enyl)-cyclohexanone	34.10^{a}	13.48^{ab}	20.49^{ab}	6.51^{b}	6.66^{b}	18.73^{ab}	0.012790
3-Hexanone	79.28^{a}	20.71^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.000019
Acetone	18.89	19.99	22.71	11.59	26.79	0.00	0.051032
Acetophenone	57.75^{a}	33.35^{ab}	8.32^{ab}	0.56^{b}	0.00^{b}	0.00^{ab}	0.014819
Cyclohexanone	43.57	56.42	0.00	0.00	0.00	0.00	0.093783
Cyclohexanone, 5-methyl-2-(1 -m*	0.00^{ab}	45.09^{a}	39.04^{ab}	0.00^{b}	15.85^{ab}	0.00^{ab}	0.008194
Pyridine, 2,4,6-trimethyl	6.03	93.96	0.00	0.00	0.00	0.00	0.086751
Aniline	18.58	12.36	22.95	11.11	21.02	13.96	0.216840
Cyanamide, dibutyl-	0.00	27.67	0.00	48.88	23.44	0.00	0.074050
Benzenamine, 3-(trifluoromethyl)-	42.55^{a}	18.87^{ab}	20.90^{ab}	4.16^{b}	13.49^{ab}	0.00^{b}	0.023143
Benzenamine, N-ethyl-	21.21^{a}	21.73^{a}	12.68^{b}	10.26^{b}	22.34^{a}	11.75^{b}	0.000056
1,2,4- Triazol-4-amine, N-(2-thien*	17.57	19.88	14.03	15.41	16.21	16.87	0.453357
2,4-Heptadienal, (E ,E)-	0.00	0.00	0.00	100.00	0.00	0.00	0.174924
2-Decene, 7-methyl-, (Z)-	0.00^{b}	24.77^{ab}	23.99^{ab}	44.73^{a}	6.50^{b}	0.00^{b}	0.004613
4-Decene, 3-methyl-, (E)	14.29	27.21	20.02	14.35	12.99	11.11	0.892779
D-Limonene	29.61^{b}	0.00^{b}	0.00^{b}	0.00^{b}	70.38^{a}	0.00^{b}	0.000004
Naphthalene	38.79	15.29	36.87	0.00	9.03	0.00	0.142601
Benzene, 1,3-bis(1,1-dimethylet**	20.29	14.18	16.06	18.27	15.63	15.54	0.737219

[abc]Different letters indicate statistical significance according to ANOVA model followed by post hoc Tuckey's HSD test ($P < 0.05$). **Cyclohexanol, 3,3,5-trimethyl-, acetate; *Cyclohexanol, 5-methyl-2-(1-methylethyl)-, cis-; *1,2,4- Triazol-4-amine, N-(2-thienylmethyl)-; ** Benzene, 1,3-bis(1,1-dimethylethyl)-.

Table S2. MANOVA categorical descriptors for the volatilome, categorized for the time of fermentation. % of contribution of VOCs descriptors significant among the food matrices.

VOCs	**Matrix**			***p value***
	Baseline	**T1**	**EP**	
Acetic acid	4.95	38.34	56.70	0.324781
Propanoic acid	8.09	30.28	61.61	0.165291
Butanoic acid	9.42	37.92	52.64	0.290858
Pentanoic acid	7.81	30.93	61.25	0.094062
Hexanoic acid	0.00	24.37	75.62	0.123153
Butanoic acid, 3-methyl-	6.76[ab]	20.55[b]	72.67[a]	0.021993
Pentanoic acid, 3-methyl-	0.00	0.00	100.00	0.056058
Ethyl Acetate	59.78[a]	27.49[ab]	12.75[b]	0.010023
Benzoic acid, methyl ester	70.04[a]	19.09[b]	10.85[b]	<0.000001
Cyanic acid, phenyl ester	17.69	65.63	16.67	0.337261
Oxalic acid, cyclohexylmethyl tetradecyl ester	0.00	5.70	94.29	0.351089
1-Hexanol	41.65	33.89	24.45	0.692706
1 -Hexanol, 2-ethyl-	39.43	40.25	20.31	0.232682
1- Propanol	21.95	35.38	42.65	0.561739
1-Butanol	26.05	39.73	34.20	0.755540
1-Heptanol	27.67	34.88	37.44	0.983888
1-Nonanol	0.00	54.45	45.54	0.373119
1-Octanol	37.08	33.10	29.81	0.925880
1-Pentanol	20.48	28.06	51.45	0.430636
Ethyl alchol	15.42	50.00	34.57	0.263346
Ethanol, 2,2'-oxybis-	25.65	39.52	34.82	0.933134
Cyclohexanol, 3,3,5-trimethyl-, ac**	24.44	42.70	32.85	0.245528
Cyclohexanol, 5-methyl-2-(1-m*	83.75[a]	0.64[b]	15.60[b]	0.000588
Benzothiazole	27.41	39.51	33.07	0.503521
Phenol	11.11[ab]	14.45[b]	74.42[a]	0.041239
Phenol, 2,4-bis(1,1-dimethylethyl)-	14.51	44.14	41.33	0.243872
p-cresol	51.13	26.16	22.69	0.074084
Phenylethyl Alcohol	19.17	8.80	72.01	0.240950
Indole	28.20	33.90	37.89	0.965925
1H-Indole, 3-methyl-	0.00	52.30	47.70	0.745936
Butanal	0.00	26.66	73.33	0.044235
Butanal, 2-methyl-	36.18	31.72	32.08	0.978824
Butanal, 3-methyl-	20.71	36.56	42.72	0.798847
Propanal, 3-(methylthio)-	4.06	46.21	49.72	0.463843
2-Nonenal, (E)-	0.00[b]	53.90[a]	46.09[ab]	0.047193
2-Octenal, (E)-	42.32	24.53	33.13	0.779027
Heptanal	32.07	36.32	31.59	0.960131

Hexanal	10.79	57.55	31.64	0.516119
Nonanal	46.90	22.45	30.63	0.643156
Octanal	68.87	3.57	27.55	0.031109
Benzaldehyde	20.10	32.32	47.57	0.026375
Benzaldehyde, 2,4-dimethyl-	49.21	31.32	19.45	0.109144
Benzaldehyde, 3-methyl-	27.99	41.82	30.18	0.564001
Benzaldehyde, 4-ethyl-	36.52	40.03	23.44	0.032673
1-Phenyl-2-butanone	82.67[a]	17.32[b]	0.00[b]	0.002860
2,3-Butanedione	90.40[a]	6.63[b]	2.95[b]	<0.000001
2-Acetylcyclopentanone	0.00	59.94	40.05	0.451552
2-Butanone	27.97	33.20	38.82	0.416244
2-Hexanone	0.00	0.00	100.00	0.192973
3-(But-3-enyl)-cyclohexanone	57.58[a]	25.26[b]	17.14[b]	0.013855
3-Hexanone	89.59[a]	10.40[b]	0.00[b]	0.000002
Acetone	34.39	33.47	32.13	0.986597
Acetophenone	75.46[a]	19.69[b]	4.83[b]	0.014455
Cyclohexanone	63.46	36.53	0.00	0.185986
Cyclohexanone, 5-methyl-2-(1 -m*	0.00	42.93	57.06	0.432203
Pyridine, 2,4,6-trimethyl	12.63	2.48	84.88	0.327151
Aniline	35.97	37.66	26.36	0.304397
Cyanamide, dibutyl-	0.00	73.79	26.20	0.143547
Benzenamine, 3-(trifluoromethyl)-	62.50[a]	18.03[b]	19.46[b]	0.026473
Benzenamine, N-ethyl-	39.56	30.40	30.03	0.545638
1,2,4- Triazol-4-amine, N-(2-thien*	34.83	34.62	30.54	0.529196
2,4-Heptadienal, (E ,E)-	0.00	0.00	100.00	0.337143
2-Decene, 7-methyl-, (Z)-	0.00	55.99	44.00	0.282931
4-Decene, 3-methyl-, (E)	28.64	42.98	28.37	0.689252
D-Limonene	18.17	33.19	48.62	0.701412
Naphthalene	58.78	25.34	15.87	0.332863
Benzene, 1,3-bis(1,1-dimethylet**	38.83	31.74	29.42	0.422822

[abc]Different letters indicate statistical significance according to ANOVA model followed by post hoc Tuckey's HSD test ($P < 0.05$). **Cyclohexanol, 3,3,5-trimethyl-, acetate; *Cyclohexanol, 5-methyl-2-(1-methylethyl)-, cis-; *1,2,4- Triazol-4-amine, N-(2-thienylmethyl)-; ** Benzene, 1,3-bis(1,1-dimethylethyl)-.

Table S3. Baseline values of beneficial VOCs in mg/kg.

VOCs	**Baseline (mg/kg)**		
Acetic acid	0.308	±	0.025
Propanoic acid	0.223	±	0.009
Butanoic acid	0.691	±	0.275
Pentanoic acid	0.352	±	0.250
Hexanoic acid	n.d.		

Table S4. Baseline values of detrimental VOCs in mg/kg.

VOCs	**Baseline (mg/kg)**		
Indole	11.79	±	7.84
1H-Indole, 3-methyl-	n.d.		
Phenol	0.37	±	0.34
p-Cresol	4.77	±	0.19

Table S5. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	**Target**	**Sequenza 3'-5'**	**Bp**	**Reference**
Eubacteria	V3-V4 16 S	Eub518-R: ATTACCGCGGCTGCTGG Eub338-R: ACTCCTACGGGAGGCAG	147	Lane *et al*, 1992
Bacteroidetes	V3-V4 16 S	Bact934F: GGARCATGTGGTTTAATT Bact1060R: AGCTGACGACAACCATG	250	Guo *et al*, 2008
Firmicutes	V3-V4 16 S	Firm934F: GGAGYATGTGGTTTAATT Eub338R: ACTCCTACGGGAGGCAG	300	Guo *et al*, 2008
Enterobacteriaceae	V3-V4 16 S	Enterobac-f: TGCCGTAACTTCGGGAG Enterobac-r: TCAAGGACCAGTGTTCAG	450	Bartosh *et al*, 2004
E. coli (total)	FtsZ	EcFtsZ-F: GGTATCCTGACCGTTGCT EcFtsZ-R: ATACCTCGGCCCAGAACT	250	Zhou *et al*, 1994
E. coli (potentially toxigenic)	CdtB	EcdtB-IVf: CGGAACGTGAATTTCGTA EcdtB-IVr: TGCCACTGTTGGAGGTC	350	Toth et al., 2003
Lactobacillales	V3-V4 16 S	F-Lac: GCAGCAGTAGGGAATCT R-Lac: GCATTYCACCGCTACACA	340	Walter *et al*, 2001
Bifidobacteriaceae	RecA	RecAf: CGTYTCBCAGCCGGAYA RecAr: CCARVGCRCCGGTCATC	220	Masco et al., 2006
Akm. muciniphila	V3-V4 16 S	AkM1: CAGCACGTGAAGGTGGG AkM2: CCTTGCGGTTGGCTTCA	327	Guo *et al*, 2016
BPP group	V3-V4 16 S	BPP-f: GAGAGGAAGGTCCCCCA BPP-r: CGCKACTTGGCTGGTTCA	140	Pachikian *et al*, 2011
B. longum	V3-V4 16 S	Blon-f: GATTCTGGCTCAGGATGA Blon-r: CTGATAGGACGCGACCC	220	Chen *et al*, 2007
Clostridium group I	V3-V4 16 S	ClosI-F: TACCHRAGGAGGAAGCCAC ClosI-R: GTTCTTCCTAATCTCTACGCAT	148	Bartosh *et al*, 2004

Clostridium group IV	V3-V4 16 S	ClosIV-f: TTAACACAATAAGTWATC ClosIV-r: ACCTTCCTCCGTTTTGTC	400	Goldberg *et al*, 2013
ATOP group	V3-V4 16S	ATOP-F: GGGTTGAGAGACCGACC ATOP-R: CGGRGCTTCTTCTGCAG	190	Matsuki *et al*, 2004
Desulfovibrio spp.	V3-V4 16S	Dsv691-f: CCGTAGATATCTGGAGG Dsv681-r: ACATCTAGCATCCATCGT	135	Fite *et al*, 2004

*Bp: base pai

7. CASE STUDY 4: Multiunit *In Vitro* Colon Model for the Evaluation of Prebiotic Potential of a Fiber Plus D-Limonene

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7.1. Introduction

The active role of the intestinal microbiota in human physiology is widely recognized, and its importance grows rapidly in the scientific literature. In the same way, it has been demonstrated that intestinal dysbiosis, characterized by low microbial diversity, has a role in the development and maintenance of most diseases. This bacterial unbalance is able to trigger low-grade chronic inflammation that impacts gut integrity and disease development (Franceschi et al., 2018). Different human diseases have been associated with intestinal dysbiosis, including autoimmune disorders, such as thyroiditis (Zhao et al., 2018; Ishaq et al., 2018), metabolic disorders, such as obesity and type II diabetes (Cotillard et al., 2013; Qin et al., 2012), and neurological disorders, such as Parkinson (Fang et al., 2020) and Alzheimer's disease (Jiang et al., 2017). In this context, an increasing number of probiotics and prebiotics have been developed in order to modulate the intestinal microbiota, often with the main purpose of relieving GI symptoms such as diarrhea, constipation, and bloating (Ford et al., 2018) as side effects of the aforementioned diseases. The action of a prebiotic on the colon microbiota is a complex phenomenon, and for its comprehension, a complex experimental model capable of considering many different parameters of the ecology of colon microbiota is necessary. In particular, the study of certain bacterial taxa and that of healthy compounds derived from fiber degradation, namely short-chain fatty acids (SCFAs) (Sanders et al., 2019) or medium-chain fatty acids (MCFAs) (Rial et al., 2016), or harmful ones derived from proteolytic fermentation, namely Indole, skatole (Roager et al., 2018), and branched-chain fatty acids (BCFAs) (Wang et al., 2020) may represent a robust strategy. The presence of these compounds derived from fiber degradation by colon microbiota should tell if the fiber evaluated fosters those beneficial bacterial groups involved in fiber fermentation rather than those involved in harmful proteolytic fermentation. To conduct such studies, in vitro gut models are considered the gold standard because they can rapidly explain the impact of food or prebiotics on the human gut microbiota, focusing on the shift of the core microbial groups and on that of selected species as well as on changes of microbial metabolites (Nissen et al., 2020).

CASE STUDY 4: Prebiotic potential of fiber plus D-Limonene food supplement

Among natural bio-actives, essential oils (EOs) from aromatic plants and their main components such as Limonene, Thymol, Piperine, Cinnamaldehyde, and Eugenol have been studied for their antimicrobic and bacteriostatic activities and have been shown to be able to modify intestinal microbiota (Spisni et al., 2020). Specifically, orange EO and its most represented component, D-Limonene, were tested in preclinical experiments in mice with promising results on the modulation of gut microbiota (Wang et al., 2019). In obesity-related disorders, orange EO showed promising preclinical data since it was able to reduce body-weight gain (Li et al., 2019), confirming D-Limonene as the active component of the oil. The same molecule efficiently reduced insulin resistance and liver damage in obese rats (Santiago et al., 2012) and counteracted dyslipidemia and hyperglycemia induced by a high-fat diet (HFD) (Li et al., 2013). Of particular interest, on the gut microbiota of obese rats, the capability of orange EO to foster *Bifidobacterium* was associated with anti-obesity proprieties. In this study, D-Limonene, which is generally recognized as safe and used in foods as a flavoring agent, was titrated at more than 97% (Li et al., 2019). Although the amounts necessary to produce beneficial effects in the host could be relevant, there are data on humans regarding the safety of chronic use of high doses of D-Limonene (Sun et al., 2007). To avoid any toxic effects from absorption of the EO in the gut and raise its effect on gut microbiota, we formulated fiber plus D-Limonene supplement (FLS), a prebiotic mixture based on D-Limonene adsorbed on cocoa fiber. In this work, to study the potential prebiotic effect of FLS, we adopted MICODE, an in vitro gut model of the distal colon, to mimic the effect of human colon fermentation. In line with the latest definition of prebiotics (Gibson et al., 2017), the use of an in vitro colon model sets the basis to study the prebiotic potential of foods while, at the same time, assessing the principal bacterial taxa and the volatilome (Nissen et al., 2020). The study of the volatilome generated during colonic fermentation of fiber is another fundamental aspect to study the prebiotic potential of a particular food or fiber because it can describe hundreds of compounds, including those derived from microbial metabolism (organic acids), and those transformed by the microbiota (bioactives) (Nissen et al., 2021; Gibson et al., 2017). In particular, in the present work, the most important bacterial taxa and their metabolites were studied by a qPCR and SPME-GC-MS, respectively. We have selected those taxa related to fiber degradation and prebiotic activity, e.g., *Bifidobacteriaceae*, as well as opportunistic taxa that should be contained by the effect of the D-Limonene, e.g., *Enterobacteriaceae*. These taxa and their shifts were analyzed based on absolute quantifications, also assaying the prebiotic index. The changes of the volatilome and the principal compounds are related to the prebiotic effect. Lastly, correlations among metabolites and bacterial taxa were also considered to better explain the interactive effects on prebiotic potential activity.

7.2. Materials and methods

7.2.1. Composition of FLS

FLS was provided by TGD Srl (Bologna, Italy). It was prepared by adsorbing pure D-limonene (>97%, Merck, Kenilworth, NJ, USA) on cocoa fiber. FLS does not contain any other ingredients or additives besides cocoa fiber and D-limonene. The final con- centration of D-Limonene in FLS was 14%. This food supplement has been patented (Patent application EP 3097921) and registered with the commercial names Limenorm® and ThangeComplex®. The analysis of its alimentary fiber content and composition was conducted by a certified external laboratory (Meriux Nutrisciences, Chelab Srl, Resana, Italy) with the official method AOAC 991.43 1994. FLS has a content of Total Alimentary Fiber of 54.0 ± 5.7 (g/100 g), composed of 43.1 ± 4.5 (g/100 g) of insoluble part and 10.9 ± 1.2 (g/100 g) of soluble part.

7.2.2. Fecal donors

Fecal donations were obtained from three healthy donors, two females and one male that respected the inclusion criteria as previously reported (Connoly et al, 2012; Koutsos et al., 2017; Oba et al., 2020). Fecal samples were collected, and processed as previously described (Nissen et al., 2021a; Nissen et al., 2021b).

7.2.3. *Materials*

Chemicals for *in vitro* colonic fermentation were of the highest analytical grade and were purchased from Oxoid (Thermo Fisher Scientific, Waltham, MA, USA), Sigma-Aldrich (St. Louis, MO, USA), and Carlo Erba Reagents (Val de Reuil Cedex, France), unless otherwise stated. Reagents for molecular biology (PCR and qPCR) as well as kits for DNA extraction and genetic standards purifications were purchased from Thermo Fisher Scientific (USA).

7.2.4. Fecal Batch-Culture Fermentation and Samples Collection

Colonic fermentations were conducted for 24 h in independent vessels on 1% (*w*/*v*) of FLS, on 1% (*w*/*v*) of fructooligosaccharides (FOS) from chicory (positive control), and on a blank substrate (blank control), using MICODE. The temperature of the model was set at 37 °C and stirred at 100 rpm, while pH was adjusted to 6.75 and maintained throughout the experiment with the automatic addition of filtered NaOH or HCI (0.5 M) to mimic the conditions located in the distal region of the human large intestine. Once the exact environmental settings were reached, the three vessels were aseptically

injected with 10 mL of fecal slurry (10% *w/v* of human feces) to a final concentration of 1% (*w*/*v*) and then two independently with 1 g of FLS or FOS for a final concentration of 1% (*w*/*v*) (Koutsos et al., 2017), while the third vessel was set as a blank control (BC) with no additives (basal medium and 1% fecal slurry only). Batch cultures were run under these controlled conditions for a period of 26.26 h, during which samples were collected at 4 time points. Fermentations were conducted in two independent experiments, using for each a new pool of feces from the same three healthy donors.

7.2.5. Pipeline of Experimental Activities

Parallel and independent vessels for FOS, FLS, and BC were run for 24 h after the adaptation of the fecal inoculum, defined as the baseline (BL). BL was defined on the first pH changes detected by Lucullus (1 read/10 s) via the pH Sensors of MICODE. For this work, the BL was set after 2.26 ± 0.15 h. The entire experiment consisted of 24 cases (n = 24), including 3 theses (FOS, FLS, and BC) and 4 time points (BL, 6 h, 18 h, and 24 h) in duplicate. Samples of the different time points were used for qPCR and SPME GC-MS analyses. After sterile sampling of 5 mL of bioreactor contents, samples were centrifuged at 16,000× *g* for 7 min to separate the pellets and the supernatants, which were used for bacterial DNA extraction and SPME-GC-MS analysis. Specifically, microbial DNA extraction was conducted just after sampling so as not to reduce *Firmicutes* content. Sampling from DNA samples and SPME-GC-MS samples were then stored at −80 °C. Technical replicas of analyses were conducted in duplicate for SPME GC-MS (n = 48) and in triplicate for qPCR (n = 72), both from two independent experiments. Statistical analyses are also reported later in detail.

7.2.6. Volatilome Analyses by SPME-GC-MS

Volatile organic compounds (VOCs) evaluation was performed on a Gas Chromatograph (7890A , Agilent, Santa Clara, CA, USA) Mass Spectrometer (5975, Agilent, USA) operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME (Solid Phase Micro-Extraction) GC-MS protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Nissen et al., 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Preconditioning, absorption, desorption of SPME–GC-MS analysis, and all data processing were carried out according to literature (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021). Molecules Identification was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). The VOCs were then sorted and super-normalized for respective chemical class, i.e., organic acids, alcohols, and other VOCs (Nissen et al., 2021a). In samples at BL the main microbial metabolites related to fermentation of

foods were absolutely quantified in mM by SPME GC-MS and an internal standard (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021a; Di Cagno et al., 2011; Casciano et al., 2021).

7.2.7. Enumeration of Bacterial Groups by qPCR

DNA was extracted from each sample at the baseline and at the different time points using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) soon after sampling. Nucleic acid purity was evaluated on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK). Changes in Eubacteria kingdom, Firmicutes and Bacteroidetes phyla, Lactobacillales order, Bifidobacteriaceae and Enterobacteriaceae families, Clostridium group I and Clostridium group IV, and Escherichia coli, Faecalibacterium prausnitzii, and Akkermansia muciniphila species were also assessed by qPCR targeting a small fragment of mono copies or multi copies genes by degenerated or specific MALDI grade primers pairs and high-fidelity DNA polymerase (Invitrogen Platinum SuperFi II DNA Polymerase, Thermo Fisher Scientific, USA) (Table S2). qPCR analyses were performed on a RotorGene 6000 (Qiagen, Hilden, Germany) with the SYBR Green I chemistry. Genetic standards were prepared from relative PCR amplicons of the target bacterial species, using a GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, USA) as described previously (Gibson et al., 2017; Nissen et al., 2019; Tanner et al., 2014; Nissen et al., 2020). For each of the targets, qPCR reactions were set as follows: a holding stage at 98 °C for 6 min, and a cycling stage of 95 °C for 20 s and 60 °C for 60 s, repeated 45 times, followed by melting curves analysis. Quantifications were made with five-points standards of the given amplicon separately. Reactions were prepared with 1 ng of DNA, 2x Power up SYBR Green (Thermo Fisher Scientific, USA) and 250 nM of each MALDI grade primers (Eurofins Genomics, Ebersberg, Germany). Details of primers pairs for PCR and qPCR, as well as qPCR performances, are supplied as Supplementary Materials (Table S2). All results were expressed as mean values obtained from triplicates from two independent experiments.

7.2.8. Prebiotic Index

The Prebiotic Index was revised from the original equation elaborated by Palframan et al. (2003), introducing substitution on bacterial taxa, the molecular approach based on quicker qPCR, data normalization, sextuplicate values, and significant differences. Analogously to the original method, an equation based on quantification values expressed as Log_{10} cell/mL are similar to the conditions applied in fermentation (24 h controlled batch with 1% *w/v* of prebiotic fiber). In this work we introduce the qPI (qPCR Prebiotic Index) based on qPCR data and this equation: qPI =

(*Bifidobacteriaceae*/Eubacteria) − (*Enterobacteriaceae*/Eubacteria) + (*Lactobacillales*/Eubacteria) − (*Clostridium* group I/Eubacteria).

7.2.9. Data Processing and Statistical Analysis

For the volatilome, one-way ANOVA ($p < 0.05$) was used to determine significant VOCs in the dataset. The dataset included 9456 interactions generated between 197 de- pendent variables (VOCs) and 48 independent variables (2 technical and 2 experimental replicas of 3 different fermentation treatments, FLS, FOS, BC (blank control), and 4 different time points, BL, 6 h, 18 h, and 24 h). The significant VOCs (n = 113) were divided into three groups and analyzed differently: (i) the prebiotic-related VOCs (preVOCs), (ii) the alkenes, (iii) the remaining volatiles. The analyses conducted were: Principal Component Analysis (PCA) to distribute the results on a plane, Multivariate ANOVA (MANOVA) to address specific contributes by categorical predictors, Tukey's HSD test for post hoc comparison. For the core microbiota, the dataset was made by 12 dependent (bacterial taxa and F/B) and 72 independent variables (3 technical and 2 experimental replicas of 3 different fer- mentation treatments, FLS, FOS, and BC, and 4 different time points, BL, 6 h, 18 h, and 24 h). It was processed for post hoc comparison by Tukey's HSD test ($p > 0.05$), as well as for the normalized dataset of qPI values. To address specific correlations among bacteria and molecules (preVOCs), two independent datasets were merged and computed by Spearman Rank analysis and visualized with a two-way joining heatmap, including Pearson dendrograms with complete linkage. The baselines of values (BL) for the volatilome and the core microbiota were obtained from the fecal slurry diluted in PBS, and the BM with the 1% (w/v) of substrates (FLS and FOS) after adaptation in the bioreactors and was expressed as the mean of three samples (Nissen et al., 2021). Normalization of datasets was performed with the mean centering method. Statistics and graphics were made with Statistica v.8.0 (Tibco, Palo Alto, CA, USA), but the two-way joining heatmap graphic was performed with Expression tool on www.heatmapper.ca (accessed on 19 July 2021).

7.3. Results

7.3.1. *Quality Controls for the Validation of MICODE*

To validate the MICODE in vitro model in the version of a fecal batch of the human distal colon, we choose to monitor and check some parameters as quality controls (Gibson et al., 2017; Takagi et al., 2016), other than the trends of the experimental conditions that were plotted over the experiments by Lucullus 3.1 (Applikon Biotechnology BV, Delft, The Netherlands). Quality controls were both related to metabolites and microbes at the end of fermentations and in comparison to BL. Specifically, (i) the *Firmicutes* to *Bacteroidetes* ratio (F/B), which is related to health and disease (Koliada et al.,

2017), was maintained low, confirming the capacity to simulate throughout the 24 h a healthy in vivo condition. (ii) The paradigm of prebiotics was confirmed. In fact, a surge in beneficial bacteria and SCFAs while a minimal depletion of enteropathogens was recorded when FOS was applied on MICODE. (iii) Each SPME GC-MS analysis had quantified some stool-related compounds (Thiourea, 1-Propanol, and Butylated hydroxy toluene) that ranged the complete chromatogram and were adsorbed at the same retention times.

7.3.2. Volatilome Analysis through SPME GC-MS

Through SPME GC-MS, among 24 duplicated cases (n = 48), 197 molecules were identified with more than 80% of similarity with the two mentioned databases. On average, 89 were relatively quantified at the baseline, while 90, 97, and 124 during the 24 h of the experiments at different time points, for BC, FOS, and FLS, respectively. 113 VOCs resulted significant by ANOVA ($p < 0.05$), which we used to describe the volatilome (Figure S1). These VOCs were sorted for the chemical class, and the sums of each class were studied as changes in respect to the baseline (Figure 1). The datasets of preVOCs (n = 14), such as SCFAs (n = 3), MCFAs (n = 6), BCFAs (n = 3), and indoles (n = 2) were super-normalized and discussed as shifts over time points (Figures 2 and Figure 3) in respect to absolutely quantified BL values (Table S1). All other VOCs sorted by chemical class, i.e., aldehydes (n = 8), ketones (n = 22), alcohols (n = 17), phenolics and sulphurates (n = 17), and alkenes (n = 30), were submitted to multivariate analyses, such as untargeted PCA (Figures 4 and Figure 5) and targeted MANOVA (Tables S3 and Table S4). Five minor compounds were cast out. Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing one chemical class to another (Nissen et al., 2020c).

CASE STUDY 4: Prebiotic potential of fiber plus D-Limonene food supplement

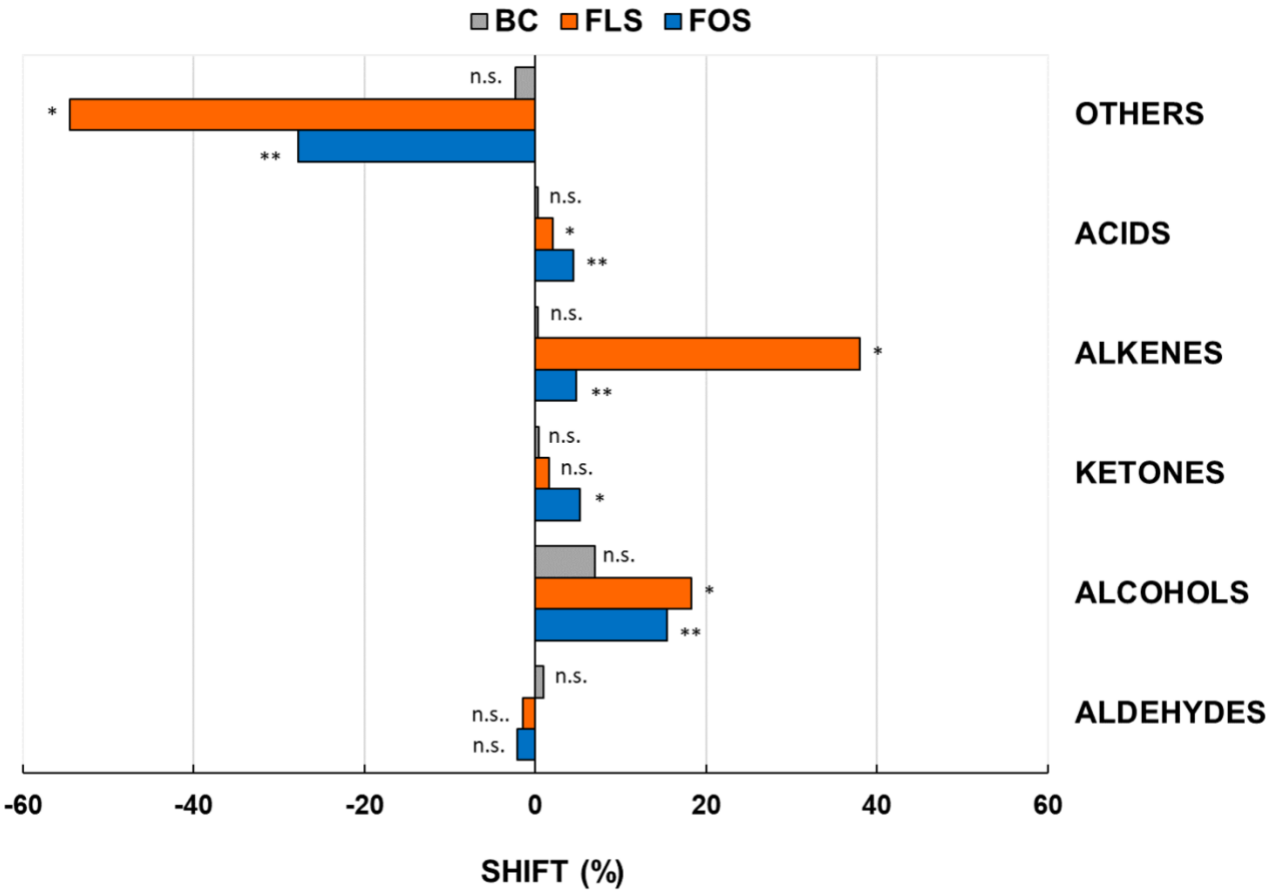

Figure 1. End point changes in VOCs expressed as relative abundances in respect to the baseline. Changes were recorded after 24 h of in vitro batch human colonic fermentations with 1% (w/v) of FLS or FOS and in the blank control. FOS = fructooligosaccharides; FLS = tested fiber; BC = blank control. *,** Significant differences by Tukey's HSD test within a chemical group ($p < 0.05$). n.s. = not significant by Tukey's HSD test ($p > 0.05$). "Others" includes phenolics and sulphurates VOCs. Samples were analyzed in duplicate from two independent experiments (n = 4).

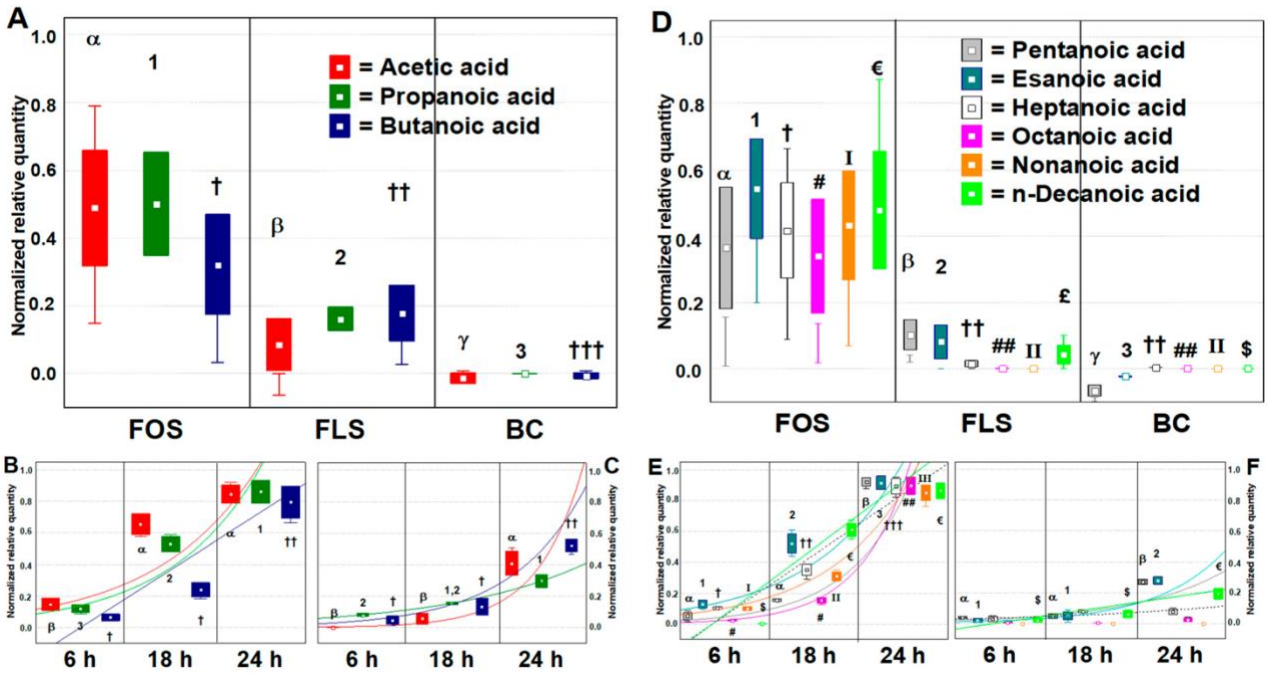

Figure 2. Changes in main microbial metabolites related to prebiotic activity expressed as a normalized scale from relative abundances in respect to the baseline value (BL = 0.0). FOS = fructooligosaccharides; FLS = tested fiber; BC = blank control. The bigger plots show changes over gathered time points, while the smaller plots show changes after specific time points, including tendency lines. The baseline absolute quantifications in mg/kg are found in the Supplementary Materials (Table S1). Changes were recorded over and after 24 h of in vitro batch human colonic fermentations with 1% (w/v) FOS and FLS. Samples were analyzed in duplicate from two independent experiments (n = 4). Boxes = mean; Rectangles = mean ± S.D.; Whiskers = Non outlier range. Cases with different Greek letters, numbers, or symbols among a single dependent variable are significantly different by Tukey's HSD test ($p < 0.05$). (A) Shifts of Short Chain Fatty Acids (SCFAs) during the fermentation time; (B) Shifts of SCFAs with FOS by time points; (C) Shifts of SCFAs with FLS by time points. Acetic acid ($p = 0.0047$); Propanoic acid ($p = 0.0226$); Butanoic acid ($p = 0.0455$). (D) Shifts of Medium Chain fatty Acids (MCFAs) during the fermentation time; (E) Shifts of MCFAs with FOS by time points; (F) Shifts of MCFAs with FLS by time points. Pentanoic acid ($p = 0.0428$); Hexanoic acid ($p = 0.0016$); Heptanoic acid ($p = 0.0043$); Octanoic acid ($p = 0.0440$); Nonanoic acid ($p = 0.0073$); n-Decanoic acid ($p = 0.0093$).

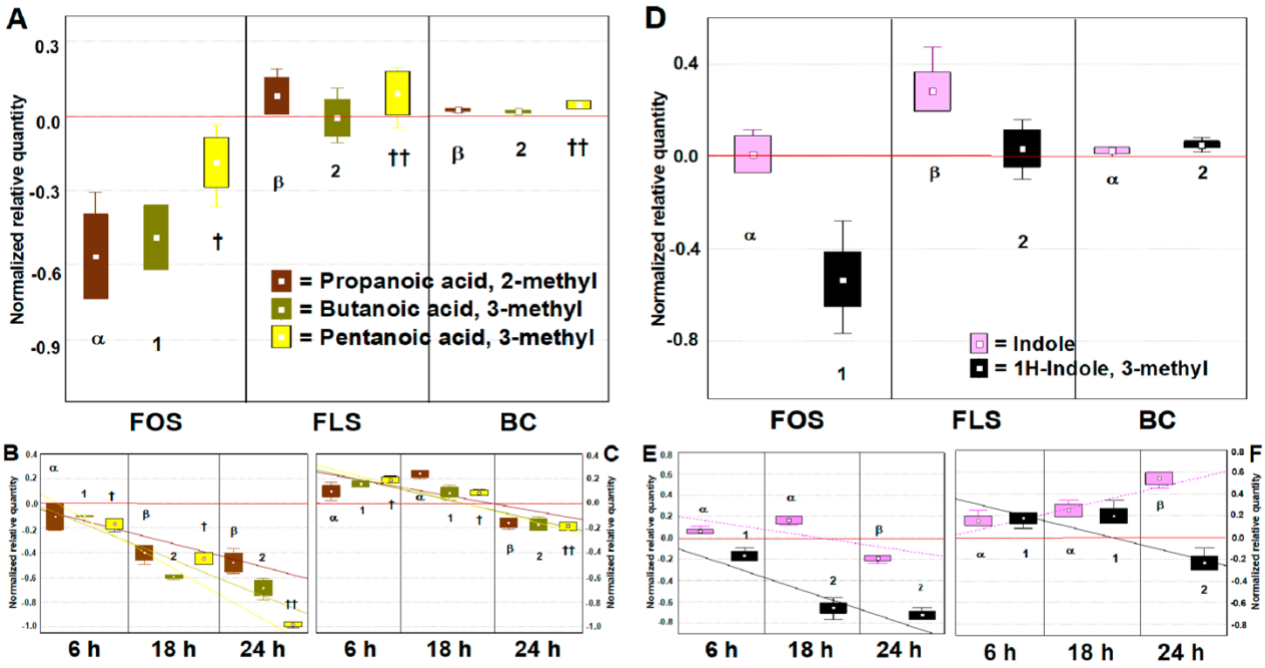

Figure 3. Changes in main microbial metabolites related to prebiotic activity expressed as a normalized scale from relative abundances in respect to the baseline value (BL = 0.0). FOS = Fructooligosaccharides; FLS = tested fiber; BC = Blank control. The bigger plots show changes over gathered time points, while the smaller plots show changes after specific time points, including tendency lines. The baseline absolute quantifications in mg/kg are found in the Supplementary Materials (Table S1). Changes were recorded over and after 24 h of in vitro batch human colonic fermentations with 1% (w/v) FOS and FLS. Samples were analyzed in duplicate from two independent experiments (n = 4). Boxes = mean; rectangles = mean ± S.D.; whiskers = non outlier range. Cases with different Greek letters, numbers, or symbols among a single dependent variable are significantly different by Tukey's HSD test ($p < 0.05$). (A) Shifts of Branched Chain Fatty Acids (BCFAs) during the fermentation time; (B) Shifts of BCFAs with FOS by time points; (C) Shifts of BCFAs with FLS by time points. Propanoic, 3-methyl acid ($p < 0.0011$); Butanoic, 3-methyl acid ($p < 0.0012$); Pentanoic, 3-methyl acid ($p < 0.0474$). (D) Shifts of indoles during the fermentation time; (E) Shifts of indoles with FOS by time points; (F) Shifts of indoles with FLS by time points. Indole ($p < 0.0330$); 1H-Indole, 3-methyl ($p < 0.0007$).

CASE STUDY 4: Prebiotic potential of fiber plus D-Limonene food supplement

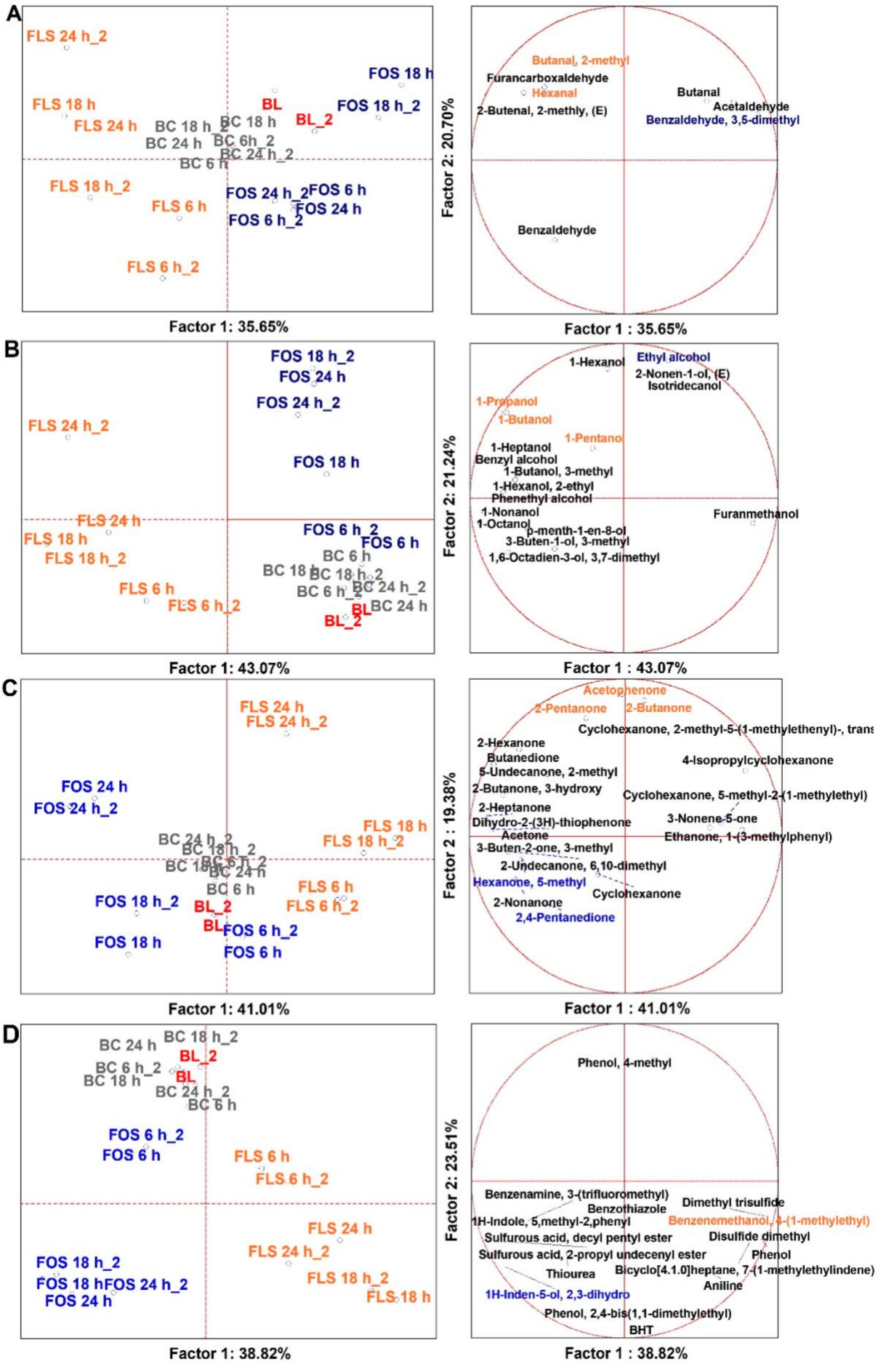

Figure 4. Changes PCAs of the volatilome sorted by chemical classes of significant (ANOVA $p < 0.05$) VOCs, including 7 duplicated cases (n = 14), as FOS and FLS fermentations at three different time points (6 h, 18 h, and 24 h) and the baseline (BL). (A) = Aldehydes; (B) = Alcohols; (C) = Ketones; (D) = Others (phenolics, sulphurates, amines). FOS = Fructooligosaccharides; FLS = tested fiber; BC = Blank control. Values were recorded over and after 24 h of in vitro batch human colonic fermentations with 1% (w/v) FOS and FLS. Samples were analyzed in duplicate from two independent experiments (n = 4). Left-side diagrams are for PCAs of cases, while right-side diagrams are for PCAs of variables. In the PCAs of variables, the variables with different font colours are the principal descriptors of FLS (orange) and FOS (blue).

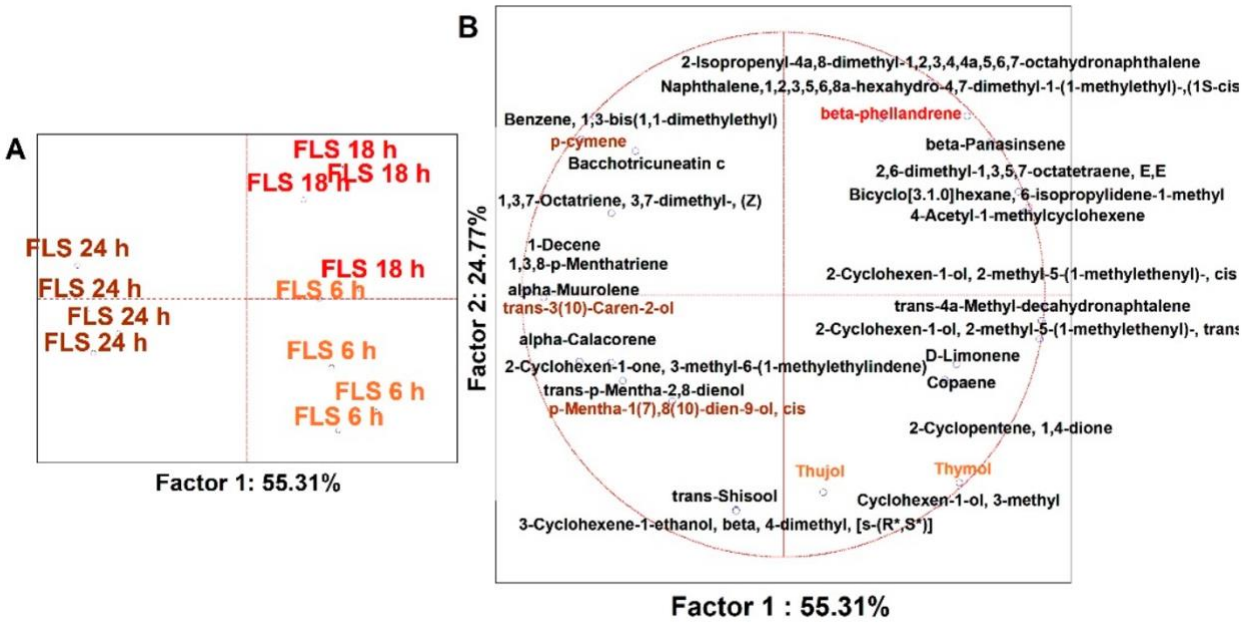

Figure 5. PCAs of the volatilome sorted by alkenes chemical classes of significant (ANOVA $p < 0.05$) VOCs, including just FLS cases (with technical replicas, n = 4) and three different time points. Values were recorded over and after 24 h of in vitro batch human colonic fermentations with 1% (w/v) FLS. (A) PCA of cases; (B) PCA of variables. In (B), the variables with different font colors are the principal descriptors of FLS cases (pale red = FLS 6 h, red = FLS 18 h, dark red = FLS 24 h).

7.3.2.1.Changes of Summarized Chemical Classes of VOCs

The 113 significant VOCs were presented as a quantification heatmap (Figure S1) for BC, FOS, and FLS cases and the BL. By Pearson dendrograms, these cases were clustered in three groups: (i) the BL and the BC samples; (ii) FLS early and intermediate time points; (iii) any FOS time points and FLS at the end point. Afterwards, these 113 VOCs were sorted by chemical class, and the sums of VOCs for each class were measured at the end point in respect to BL (Figure 1). No differences were detected for BC samples in respect to BL ($p < 0.05$). Significant changes were observed for FOS and FLS fermentations in respect to BL, in particular: (i) phenolics and sulphurates (others), whose

abundances were reduced over time for the 28% and 54% by fermentations with FOS and FLS, respectively; (ii) organic acids, that have increased of 5% and 8% by fermentations with FOS and FLS, respectively; (iii) alkenes, that have increased largely just by fermentation with FLS (37%); (iv) ketones, that have increased either by fermentations with FOS or FLS; (v) alcohols, that have increased around 18% either by fermentations with FOS or FLS. The described metabolic shift may be ascribed to the fermentation activity of colonic bacteria, which are able to transform phenols by the action of *Lactobacillaceae* and *Eubacteriaceae* members, liberating alkenes and producing alcohols (Kemperman et al., 2013).

7.3.2.2. VOCs Related to Prebiotic Activity (preVOCs)

To analyze the main changes in volatile microbial metabolites related to prebiotic potential, we considered the shift (compared to BL) to the different time points of fermentation of 14 selected VOCs. These VOCs were related to prebiotic activity (preVOCs) and have renowned bioactivity for the host (SCFAs, MCFAs, BCFAs, Indole, and skatole). The dataset was made by 48 independent variables (3 time points, two duplicates for FOS, FLS, and BC) that we have analyzed as follows: (a) every single compound was normalized (mean centering method) within its dataset, which included cases from different type of samples; (b) the BL dataset (Table S1) was then subtracted to the end point dataset; (c) post hoc analysis was done to compare to each other the samples' productions of a single molecule (Tukey's HSD test, $p < 0.05$). The BC was graphically included but had no differences in respect to the baseline (SCFAs, BCFAs, and Indole) or no detectability (MCFAs and skatole) ($p > 0.05$).

Short Chain Fatty Acids (SCFAs) are essential compounds for the host, the mucosa, and the colon microbiota. They contribute to cell homeostasis (Sun et al., 2018), hormone regulation in the bloodstream (Larraufie et al., 2018), counteraction of opportunistic and pathogenic bacteria (Lamas et al., 2019), and fostering probiotics and beneficial microbes (Sanders et al., 2019). From our results, every SCFAs (Figure 2A) increased either with FOS or FLS at any time points in respect to BL ($p < 0.05$), while no increments were recorded with the BC samples in respect to the baseline ($p > 0.05$). FLS delivered during fermentation up to 22.2% more SCFAs, e.g., butanoic acid, than the baseline. Although, FOS had thrice the capacity than FLS in producing SCFAs (5.7-, 3.1-, and 1.8-fold more Acetic, Propanoic, and Butanoic acid, respectively) ($p < 0.05$). Considering specific time points (Figure 2B,C), our results showed that the increment in SCFAs was little at the early and intermediate time points and had its hit at the end point, where 37.6%, 29.7%, and 48.7% higher quantities were recovered for Acetic, Propanoic, and Butanoic acids, respectively ($p < 0.05$). In literature, many reports observed that a reduction in SCFAs content is linked to a reduced eubiosis of the gut

microbiota and reduced intestinal cell homeostasis, either experienced in vivo and in vitro (Moens et al., 2019). The prebiotic potential of FLS is, therefore, mainly derived by the capacity to foster those bacteria able to deconstruct the fiber and liberate SCFAs in the colon niche, similarly to FOS.

Medium Chain fatty Acids (MCFAs) are unsaturated fatty acids (from C6 to C12) that have a beneficial effect on the host. For example, MCFAs are active in the protection of glucose homeostasis during high-fat overfeeding and are effective in conditions of insulin resistance (Lundsgaard et al., 2021). MCFAs are produced by colon microbiota during chain elongation of intermediate fermentation products of fibers (Scarborough et al., 2020) or by direct fiber degradation performed by *Bifidobacteriaceae* (Riviere et al., 2018). For example, *Enterobacteriaceae* and *Bacteroides* spp. produce MCFAs from lactate, while *Lachnospiraceae* from xylose and other pentoses (Riviere et al., 2018). In respect to the baseline, any MCFAs (Figure 2D) increased during fermentation with FOS, while just Pentanoic, Hexanoic, and n-Decanoic acids increased during fermentation with FLS ($p < 0.05$). No differences were detected for BC samples in respect to the baseline ($p > 0.05$). Generally, FOS produced four-fold more of these three compounds in comparison to FLS. Anyhow, considering specific time points (Figure 2E,F), the increments scored by FLS turned significant just at the end point, were accounted for 27.1%, 27.9%, and 19.5% more abundance of Pentanoic, Hexanoic, and n-Decanoic acids, respectively ($p < 0.05$). MCFAs are important metabolic biomarkers of Intestinal Bowel Disease (IBD)-related changes. The levels of MCFAs significantly decreased in patients with IBD. For example, Hexanoic acid levels are inversely correlated to disease activity in IBD (De Preter et al., 2015). So far, a reduction in MCFAs content should be linked to a dysbiosis of the gut microbiota.

Branched Chain Fatty Acids (BCFAs), such as Propanoic acid, 3-methyl, Butanoic acid, 3-methyl, and Pentanoic acid, 3-methyl, are derived from microbial colon protein fermentation and produce NH_3, phenol, and sulfate amines that could be stressful for the host (Aguirre et al., 2016). BCFAs are often used as a biomarker of protein catabolism, with the promoted target to reduce their concentration and improve health outcomes (Yao et al., 2016). Still, little is known about the impact of BCFAs on host health (Oliphant et al., 2019; Solon-Biet et al., 2019). What is undisputed, however, are the negative consequences of the pro-inflammatory and cytotoxic compounds yielded from the sulfur-containing, basic and aromatic amino acids (Oliphant et al., 2019; Solon-Biet et al., 2019). From recipient results, BCFAs (Figure 3A) overall increased during fermentation with FLS, but not significantly ($p > 0.05$), while they were significantly reduced during fermentation with FOS ($p < 0.05$). No differences were detected for BC samples in respect to the baseline ($p < 0.05$). Notwithstanding, considering single time points (Figure 3B,C), significant reductions of the three BCFAs were also seen at the end point, specifically, −13.2%, −17.1%, and −11.2% for Propanoic

acid, 3-methyl, Butanoic acid, 3-methyl, and Pentanoic acid, 3-methyl, respectively. Thus, at the end point, FLS was able to reduce BCFAs, but in comparison to FOS, this reduction was 3.1-, 3.9-, and 8.9- fold weaker for Propanoic acid, 3-methyl, Butanoic acid, 3-methyl, and Pentanoic acid, 3-methyl, respectively. The reduction driven by FLS at the end point could testify that our product is shaping the microbiota, fostering the growth of that core bacterial groups specialized in the fermentation of fibers, more than that specialized in protein fermentation. As this effect happens even when FOS is fermented, we can add another notch to the prebiotic potential of FLS.

Indole and skatole (1H-Indole, 3-methyl) are two compounds of tryptophan (trp) catabolism derived from the degradation of the proteinaceous portion of the food or diet. Besides trp metabolism by the host, resident microbiota can directly utilize trp (Agus et al., 2018). Different commensal bacteria catabolize trp using tryptophanase into indoles, and several different derivatives are formed (Hendrikx et al., 2019). Whereas Indole is also suggested to have beneficial effects like attenuation of inflammation indicators (Bansal et al., 2010), bacterial production (*Clostridium* spp. and *Escherichia* spp.) and its accumulation are toxic for the host because it alters permeability and homeostasis of the mucosa (Roager et al., 2018), and once it is metabolized into indoxyl sulfate in the liver, can lead to chronic kidney disease and vascular diseases (Wang et al., 2020; Hendrikx et al., 2019). Bacterial decarboxylation (*Bacteroides* spp. and *Clostridium* spp.) of trp produces harmful skatole that is associated with the production of inflammatory cytokines (Roager et al., 2018). From the results obtained (Figure 3D), in respect to the baseline dataset, the shifts recorded by FOS and FLS fermentations indicated a different trend. BC produced slight changes for both compounds in respect to the baseline ($p > 0.05$). FOS was able to significantly reduce the quantity of skatole by about 55% ($p < 0.05$), but no differences were found in Indole production ($p > 0.05$). FLS fermentations, instead, did not generate significant changes in both compounds ($p > 0.05$) but showed a slight increase in Indole. Notwithstanding, FLS was able to reduce significantly by 21.3% the production of skatole at the end point ($p < 0.05$). Thus, considering this time point (Figure 3E,F), FOS had 3.41-fold more strength to reduce harmful skatole than FLS. Modulation of trp and protein metabolism may benefit the gut host, especially when dysbiosis is involved (Hendrikx et al., 2019). Like the results obtained with BCFAs, the prebiotic potential of FLS at the end point may be ascribed to shaping the microbiota to the advantage of those bacterial groups specialized in fibers, more than in proteins fermentation.

7.3.2.3. Volatilome Analysis of Aldehydes, Ketones, Alcohols and Phenolics

Through SPME GC-MS, compounds other than those typically related to prebiotic activity were investigated. The volatilome was studied on 4 super-normalized datasets of VOCs sorted for their

chemical class, each generated from different numbers of dependent variables (VOCs) and 40 independent variables (3 time points, two duplicates for FOS, FLS, BC, and BL). These datasets were submitted to a multivariate approach, including untargeted PCA and targeted MANOVA. The BC was not included in MANOVA analyses since it had no significant variances for these molecules (ANOVA $p > 0.05$).

A PCA of 8 statistically significant aldehydes distributed cases on the plot, separating fermentation with FOS and FLS from each other and BL (Figure 4A). Principal descriptors of fermentation with FLS were Butanal, 2-methyl, and 2-Hexanal ($p < 0.01$), produced at the end point (24 h) ($p < 0.01$) (Tables S3 and S4). From our results, the contribution to aldehydes production from the BC samples remains indiscriminate ($p > 0.01$). 2-Hexanal was reported to limit the growth of several intestinal pathogens at a low concentration (Cho et al., 2004). It is conceivable that this resulted from the degradation of very-long-chain organic acids (Mitro et al., 2012) present in FLS. Aldehydes are a result of microbial fermentation and lipid oxidation, as well as the transformation of ethyl alcohol (Malaguarnera et al., 2014). Certain aldehydes are health-promoters because they contribute positively to cell homeostasis and microbiota eubiosis, such as Indole-3-aldehyde (Alexeev et al., 2018), while most are detrimental, being cytotoxic at a low threshold, such as Acetaldehyde (Na et al., 2017).

PCA of 17 statistically significant alcohols distributed cases on the plot, separating fermentation with FOS and FLS from each other and BL (Figure 4B). From our results, the contribution to alcohol production from the BC samples remains indiscriminate ($p > 0.01$), while the descriptor of fermentation with FOS was mainly Ethyl alcohol ($p < 0.01$), and those for FLS were 1-Butanol, 1-Propanol, and 1-Pentanol, mainly produced at the late time points ($p < 0.01$) (Tables S3 and S4). Alcohols are essential compounds of the fermentation of dietary polysaccharides conducted by the colon microbiota (Oliphant et al., 2019). It is reported that 1-Pentanol is associated with the consumption of old grains and has anti-inflammatory and prebiotic activity (Taneyo Saa et al., 2014).

The PCA of 22 statistically significant ketones distributed cases on the plot, separating the substrates from each other and from BL (Figure 4C). From our results, the contribution to ketones production from the BC samples remains indiscriminate ($p > 0.01$). Descriptors of fermentation with FOS was principally 2.4-Pentanedione. Descriptors of fermentation with FLS were: 2-Pentanone, 2-Butanone, and Acetophenone, largely produced at the end point ($p < 0.01$) (Tables S3 and S4). During colon fermentation, many ketones are produced. Considering their bioactivity, some are desirable, such as the ketones bodies (Cabrera-Mulero et al., 2019); others, such as acetone, are unwanted because they could be toxic for the host (Bradberry et al., 2007). Acetophenone deserves attention since it acts as an antimicrobial to different Gram-negative bacteria (Tran et al., 2020), and its N-substitute derivates

have been proposed as a therapeutic approach in diabetes (Taslimi et al., 2020). In our experimental conditions, it probably derived from the bacterial deconjugation of polyphenols, where FLS is presumably rich. A bacterial group implied in such action is *Lactobacillales* (Cardona et al., 2013), which was increased after FLS.

Considering the remaining VOCs, we have included amines, sulphurates, and phenolics (Figure 4D) and elaborated a PCA of 17 statistically significant VOCs. The distribution of cases on the plot was made by separating fermentation with FOS and FLS from each other and BL. Moreover, time-dependent discrimination evidenced that the fermentation conditions adopted in the MICODE gut model allowed larger speciation of molecules at the late time points. While the descriptor of fermentation with FOS was mainly 1H-Inden-5-ol, 2,3-dihydro ($p < 0.01$), that for FLS was Benzenemethanol, 4-(1-methylethyl) ($p < 0.01$) (Tables S3 and S4). This latter compound is known as Cuminyl alcohol and is reported to have anti-oxidant potential (Tamta et al., 2016). Lastly, even in this situation, the contribution to VOCs production from the BC samples remains indiscriminate, except for Phenol, 4-methyl ($p < 0.01$) (Tables S3 and S4). Considering Phenol, 4-methyl, it is interesting to mention that it was not a descriptor of FOS nor FLS fermentation; likely, its content was reduced from the baseline value. This VOC is associated with cardiovascular diseases and is derived from excessive proteolytic fermentation of Western diets, mainly due to species of *Ruminococcus* and *Clostridium* (Mei et al., 2020).

7.3.2.4. Time-Dependent Discrimination of Alkenes

The dataset of 30 statistically significant alkenes was almost entirely related to FLS fermentation due to its EO content; thus, no MANOVA was applied. The study was conducted on 12 dependent variables (no FOS nor BC were included but was the BL). Results are discussed just by PCA, which has distributed cases on the plot on a time basis through robust factors (Figure 5). These alkenes are complex VOCs belonging to the class of terpenes and terpenoids, with renowned bioactive features. In detail, most of the compounds were pushing the cases of early and intermediate time points to the I and IV quadrants, while minor speciation of alkenes limited the cases of the end point on the middle of the left side quadrants. Besides, the terpenes and terpenoids describing the early and intermediate samplings majorly were those famous for their strong antimicrobial activity, e.g., Thymol, beta-Phellandrene, and Thujol (Nissen et al., 2019), while the VOCs describing the end point cases possess an anti-oxidant nature, e.g., p-Cymene (Marchese et al., 2017), trans-3-(10)-Caren-2-ol (Mehmood et al., 2019), and p-Mentha-1(7),8(10)-dien-9-ol (cis-Carveol) (Hritcu et al., 2020). Lastly, it is interesting to observe even that 4-acetyl-1-Methylcyclohexene, which is a major compound derived from D-Limonene oxidation but toxic for host cells (Lipsa et al., 2018), was not a descriptor of end

point cases, meaning that it was depleted during fermentation and somehow converted by colonic bacteria.

7.3.3. Microbiota Analysis

To study the potential benefits associated with FLS beneficial effects on colon microbiota, we considered: (i) the changes of 11 core colon bacterial taxa (ii) the *Firmicutes* to *Bacteroidetes* ratio, as an indicator of eubiosis: (iii) the qPCR Prebiotic Index (qPI) based on quantification values of selected bacterial taxa over time of fermentation, in comparison to FOS and the blank control.

7.3.3.1. Changes in Selected Fecal Bacterial Populations Measured with qPCR

The changes in the core microbiota regarded Eubacteria kingdom, *Firmicutes* and *Bacteroidetes* phyla, *Lactobacillales* order, *Bifidobacteriaceae* and *Entero bacteriaceae* families, *Clostridium* group I and group IV, and *E. coli*, *F. prausnitzii*, and *A. muciniphila* species were assessed by qPCR (Table 1). At the early time point (6 h), few significant changes were found among all cases and bacterial targets ($p < 0.05$). Eubacteria and *Bifidobacteriaceae* increased during FOS fermentation, *Lactobacillales* increased either with FOS or FLS, *Clostridium* group I was augmented by any type of fermentation, but *Clostridium* group IV was reduced just by FLS ($p < 0.05$). At the intermediate time point (18 h), microbiota changes were more consistent with respect to BL. For example, *Lactobacillales* increased in number both for FOS and FLS fermentations, *Bifidobacteriaceae* just for that of FOS ($p < 0.05$), *Enterobacteriaceae* increased just in the control. Besides, while *Clostridium* group I loads increased with any fermentation, *Clostridium* group IV decreased with FLS ($p < 0.05$).

Table 1. Absolute quantification by qPCR and SYBR Green I chemistry expressed as means of sextuplicates and S.D. in Log_{10} GCN/mL *.

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	Baseline	FOS			FLS			BC		
	0 h	6 h	18 h	24 h	6 h	18h	24 h	6 h	18 h	24 h
Eubacteria	8.27 ± 0.22 b	8.61 ± 0.12 a	8.69 ± 0.16 a	8.96 ± 0.17 a	8.29 ± 0.11 b	8.41 ± 0.11 b	8.82 ± 0.12 a	8.36 ± 0.10 b	8.18 ± 0.08 b	8.34 ± 0.07 c
Firmicutes	7.37 ± 0.20 b	7.45 ± 0.07 b	7.84 ± 0.08 b	8.43 ± 0.07 c	7.54 ± 0.13 b	7.56 ± 0.14 b	8.68 ± 0.09 a	7.38 ± 0.02 b	7.35 ± 0.22 b	7.16 ± 0.15 c
Bacteroidetes	6.41 ± 0.18 b	6.61 ± 0.15 b	7.25 ± 0.13 b	8.26 ± 0.07 a	5.91 ± 0.16 b	6.14 ± 0.10 b	7.75 ± 0.18 a	6.22 ± 0.24 b	5.16 ± 0.03 c	4.77 ± 0.11 c
Lactobacillales	6.67 ± 0.13 c	7.27 ± 0.17 b	7.69 ± 0.12 a	8.23 ± 0.12 a	6.98 ± 0.23 b	7.14 ± 0.11 b	8.17 ± 0.14 a	6.55 ± 0.07 c	6.71 ± 0.11 c	6.63 ± 0.08 c
Bifidobacteriaceae	7.21 ± 0.08 b	7.71 ± 0.08 a	7.88 ± 0.04 a	7.96 ± 0.04 a	6.69 ± 0.20 b	6.69 ± 0.12 b	8.11 ± 0.17 a	6.56 ± 0.21 b	6.18 ± 0.04 b	5.20 ± 0.12 c
Enterobacteriaceae	6.77 ± 0.19 bc	6.99 ± 0.21 b	7.09 ± 0.22 b	6.31 ± 0.24 c	7.04 ± 0.07 b	6.98 ± 0.08 b	6.55 ± 0.11 c	7.10 ± 0.28 b	8.10 ± 0.28 a	8.33 ± 0.34 a
Clos group I	2.13 ± 0.29 c	3.48 ± 0.43 b	3.49 ± 0.41 b	2.35 ± 0.13 c	4.28 ± 0.27 b	4.95 ± 0.29 b	4.33 ± 0.23 b	4.20 ± 0.30 b	6.19 ± 0.30 a	6.16 ± 0.51 a
Clos group IV	7.43 ± 0.11 a	7.35 ± 0.18 a	7.33 ± 0.19 a	7.20 ± 0.16 a	6.66 ± 0.23 b	6.34 ± 0.23 b	6.57 ± 0.04 b	7.53 ± 0.19 a	7.38 ± 0.28 a	7.37 ± 0.08 a
E. coli	3.96 ± 0.06 c	4.24 ± 0.05 c	4.05 ± 0.06 c	3.81 ± 003 c	4.30 ± 0.15 c	4.11 ± 0.06 c	4.03 ± 0.05 c	5.08 ± 0.25 b	6.44 ± 0.13 a	6.79 ± 0.09 a
F. prausnitziii	7.53 ± 0.12 b	7.66 ± 0.07 b	8.05 ± 0.12 ab	8.57 ± 0.24 a	7.57 ± 0.25 b	7.13 ± 0.04 b	8.12 ± 0.15 ab	7.07 ± 0.08 bc	6.88 ± 0.05 c	6.49 ± 0.11 c
A. muciniphila	4.19 ± 0.13 b	4.87 ± 0.10 a	4.55 ± 0.18 ab	4.58 ± 0.16 ab	4.15 ± 0.07 b	4.34 ± 0.08 b	4.95 ± 0.05 a	4.53 ± 0.07 ab	3.40 ± 0.03 c	3.20 ± 0.04 c
F/B **	1.15 ± 0.20 b	1.13 ± 0.11 b	1.08 ± 0.10 b	1.02 ± 0.07 b	1.28 ± 0.14 ab	1.23 ± 0.12 b	1.12 ± 0.14 b	1.19 ± 0.13 a	1.42 ± 0.13 a	1.50 ± 0.12 a

BL = Baseline; FOS = Fructooligosaccharides; FLS = tested fiber; BC = Blank control. * GCN/mL = gene copy number/mL; ** F/B = *Firmicutes/Bacteroidetes*; a,b,c Different letters within a microbial taxon indicate statistical significance by Tukey's HSD test ($p < 0.05$). Primers pairs are shown in Table S2. Samples were analyzed in triplicate from two independent experiments (n = 6). *Clos group I = Clostridium group I*; *Clos group IV = Clostridium group IV*; *E. coli = Escherichia coli; F. prausnitziii = Faecalibacter prausnitzii*; *A. muciniphila = Akkermansia muciniphila*.

At the end point (24 h), 25 out of 30 cases scored significant changes in abundance of any bacterial targets ($p < 0.05$), including those at the species level that did not record changes previously. For example, total Eubacteria, *Bacteroidetes*, *Lactobacillales*, and *Bifidobacteriaceae* recorded increased numbers with FOS and FLS fermentations. *Enterobacteriaceae* were reduced by both FOS and FLS but not significantly for the latter. *Clostridium* group I was reduced by FOS, while it was not by FLS; nevertheless, it was almost two Log_{10} lower than the control ($p < 0.05$). Considering FOS, the substantial reduction we have observed in *Clostridium* group I and the concomitant increment in *Clostridium* group IV could be due to allolysis or other antagonistic interactions reported to happen in a closed environment and on Gram + and/or sporulating bacteria (Prozorov et al., 2011; Claverys et al., 2007; Rosenberg et al., 2016). Considering the species level, *E. coli* remained at low thresholds with FOS and FLS and surged just in the control ($p < 0.05$). *F. prausnitzii* and *A. muciniphila* were fostered either by FOS or FLS, but the former significantly by FOS, while the latter

significantly by FLS ($p < 0.05$). Our results are comparable to those obtained in literature by similar investigations in similar colon models (Connolly et al., 2012; Koutsos et al., 2017; Takagi et al., 2016), and those of FOS and FLS respect the concept of prebiotics, for which a compound must foster the growth of beneficial and probiotics bacteria (*Bifidobacteriaceae* and *Lactobacillales* or *F. prausnitzii* and *A. muciniphila*), while simultaneously reduce or contain that of opportunistic and pathogenic (*Enterobacteriaceae*, *E. coli*, and *Clostridium* group I), relatively to a healthy intestine.

7.3.3.2. Firmicutes/Bacteroidetes (F/B) over Time

To evidence the prebiotic and eubiotic potential of FLS, it is important to stress the trend of the ratio *Firmicutes*/*Bacteroidetes* (F/B) over time. This ratio indicates an eubiosis of the microbiota when ranging around and lower than 1.5, and a dysbiosis when more than 2, leading to intestinal syndromes (Koliada et al., 2017; Zhou et al., 2017). In this study, at BL, fecal samples recorded an F/B of around 1.15, indicating the healthy condition of the donors, and this ratio was similar ($p > 0.05$) after fermentation either with FOS (1.01) or FLS (1.12), while it was 1.50 when colonic fermentation was conducted with the blank control ($p < 0.05$). These findings confirmed the positive role of FLS able to increase *Bacteroidetes* proportion and limit that of *Firmicutes*, other than *Lactobacillales*.

7.3.3.3. Prebiotic Index

In this paper, we suggested qPI (qPCR Prebiotic Index) to strengthen the original Prebiotic Index equation elaborated on 24 h controlled batch culture condition with 1% *w/v* addition of prebiotic by Palframan and colleagues almost 20 years ago (Palframan et al., 2003). qPI was obtained, normalizing the data and substituting the *Bacteroides* taxon with that of *Enterobacteriaceae* because, in the former, there are many species that have been recently considered beneficial (e.g., *Bacteroides ovatus*) (Oba et al., 2020; Depres et al., 2016), while almost all members of *Enterobacteriaceae* are generally described as opportunistic and pathogenic. Moreover, the old equation was made on values obtained by the FISH technique, while we are proposing sextuplicate values obtained from the qPCR technique.

Considering the results (Figure 6), we found that the best performer was FOS after 18 h of fermentation, and the runner-up was FLS after 24 h of fermentation. In comparison to FOS 18 h, FLS fermentation scored 1.85- and 2.03-fold lower values, at 6 and 18 h time points, respectively. Otherwise, this trend was rebalanced by FLS at the end point, which was just 1.23-fold lower than FOS 18 h. The blank control scored for any time points lower values than any FOS or FLS case (all

significant, but one) and reached the lowest value of the dataset at the end point (26.24-fold lower than FOS 18 h). So far, the qPI of FLS tends to reach a high level later than the FOS. Anyhow, even at the earlier time points qPI of FLS was higher than the blank control. Thus, the comparable prebiotic index of FLS could be mostly due to its high portion of soluble fiber that accounted almost for 80%. Similarly to FOS, it is known that soluble fibers are excellent substrates to produce SCFAs in the large intestine (Bengmark et al., 2005; Slavin et al., 2013). Thus, even from the qPI outputs, it has been possible to see a slower but effective SCFAs production of FLS compared to FOS. This aspect is two-faced: a fast microbial turnover and high production of beneficial compounds are foreseen as an issue for a fiber aspiring prebiotic potential, on the other side, the capacity to slow microbial metabolism as well as to contribute to a more stable microbial yield and composition over time could address to FLS other unexpected features, such as longevity potential. It has been recently described that the well-established Tg2576 mouse model of Alzheimer's disease is described by more age-related microbiome changes in comparison to wild type that are reversible when balanced by certain nutrition regimes (Cox et al., 2019). In this way, FLS, after further research, could have the potential to target specific consumers, such as the elderly. Lastly, it must be recognized that even if the prolongation of life span is beyond this study, not recently a critical mass of scientists is arguing over the concept that a slower metabolism is able to extend life span in *Caenorhabditis elegans* (Van Raamsdonk et al., 2010).

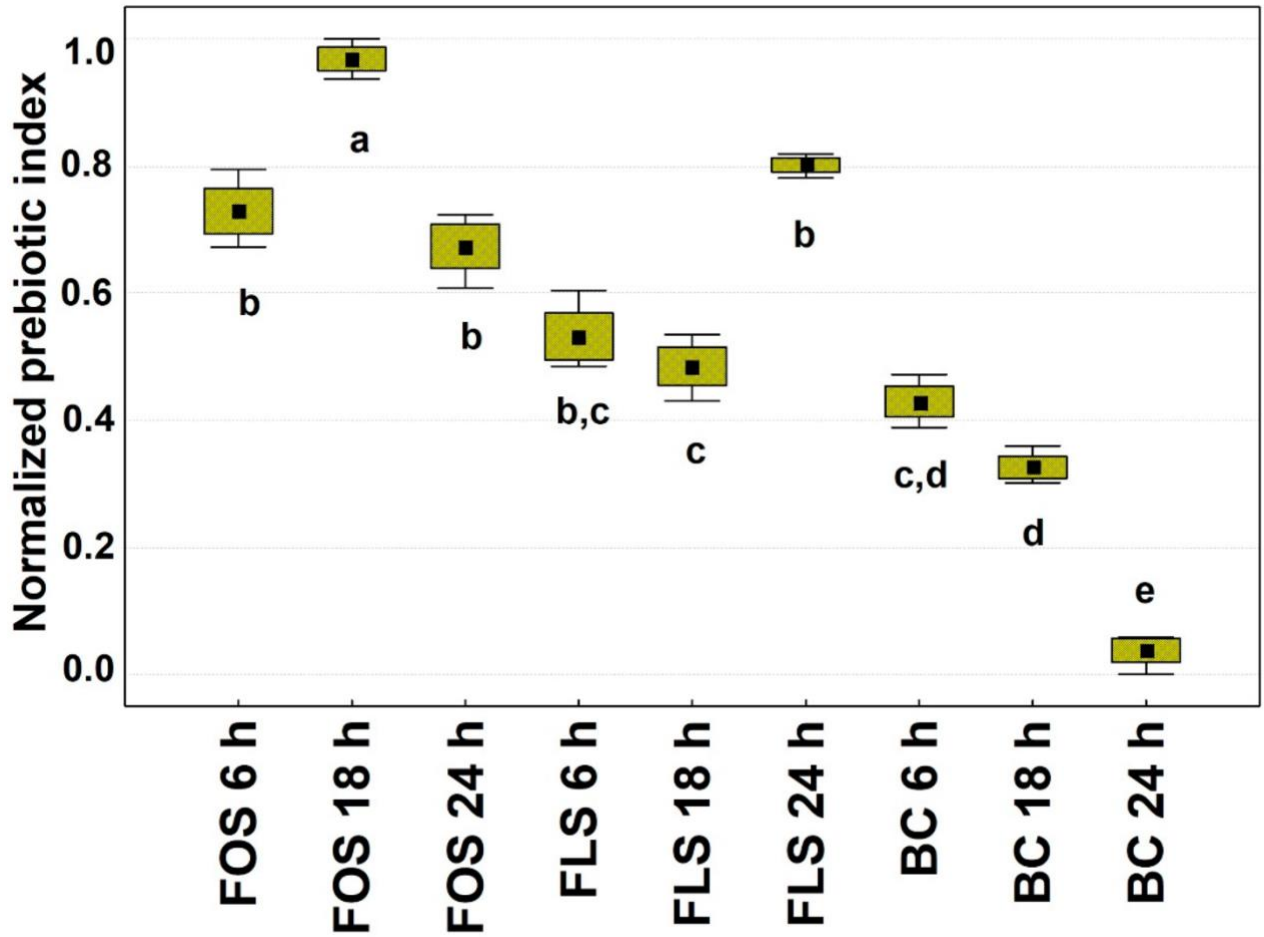

Figure 6. qPCR Prebiotic Index (qPI). FOS = Fructooligosaccharides; FLS = tested fiber; BC = Blank control. a,b,c,d,e Different letters indicate statistical significance by Tukey's HSD test ($p < 0.05$). Black squares = mean; Boxes = mean ± S.D.; Whiskers = min and max.

7.3.4. Microbiota-Metabolites Correlations over the Prebiotic Activity

Spearman Rank Correlations ($p < 0.05$), two-joining-way Heatmaps, and Pearson cluster analysis were performed among bacterial taxa and VOCs related to prebiotic activity by the comparison of two different normalized datasets, each respectively derived from quantitative datasets of qPCRs and SPME GC-MS (Figure 7 and Figure S2). Only significant correlations will be discussed. From the Pearson dendrograms on both columns and rows, two major descriptors of prebiotic potential were identified: (i) those responsible for beneficial effects and (ii) those for the detrimental. For example, in the first group, in order of significance, *F. prausnitzii*, *Lactobacillales*, *Bacteroidetes*, *Bifidobacteriaceae*, *Clostridium* group IV, and *A. muciniphila* had positive correlations to beneficial VOCs (VOCs from C2 to C10) while negative to detrimental VOCs (BCFAs and skatole). In the second group, *Enterobacteriaceae*, *E. coli*, *Clostridium* group I, and *Firmicutes* had more intense positive correlations among detrimental

VOCs as well as negative correlations among beneficial VOCs. The increased abundances in SCFAs and MCFAs in our dataset were correlated mainly to *Lactobacillales* and *F. prausnitzii*, as seen by other authors (Scarborough et al., 2020; Riviere et al., 2018). While the shorter compounds were even produced by *Bacteroidetes*, the longer compounds were correlated to *Bifidobactreiaceae*. FLS and FOS were able to foster the growth of those beneficial bacterial taxa, as well as were able to induce the production of beneficial VOCs. Similar results were already observed with FOS by several authors (Wang et al., 2020; Gibson et al., 2017; Connolly et al., 2012; Koutsos et al., 2017; Takagi ey al., 2016). Besides, FLS and FOS were able to diminish or contain the population of opportunistic *Enterobacteriaceae*, *Clostridium* group I, and *E. coli*, as well as to reduce the production of skatole and BCFAs.

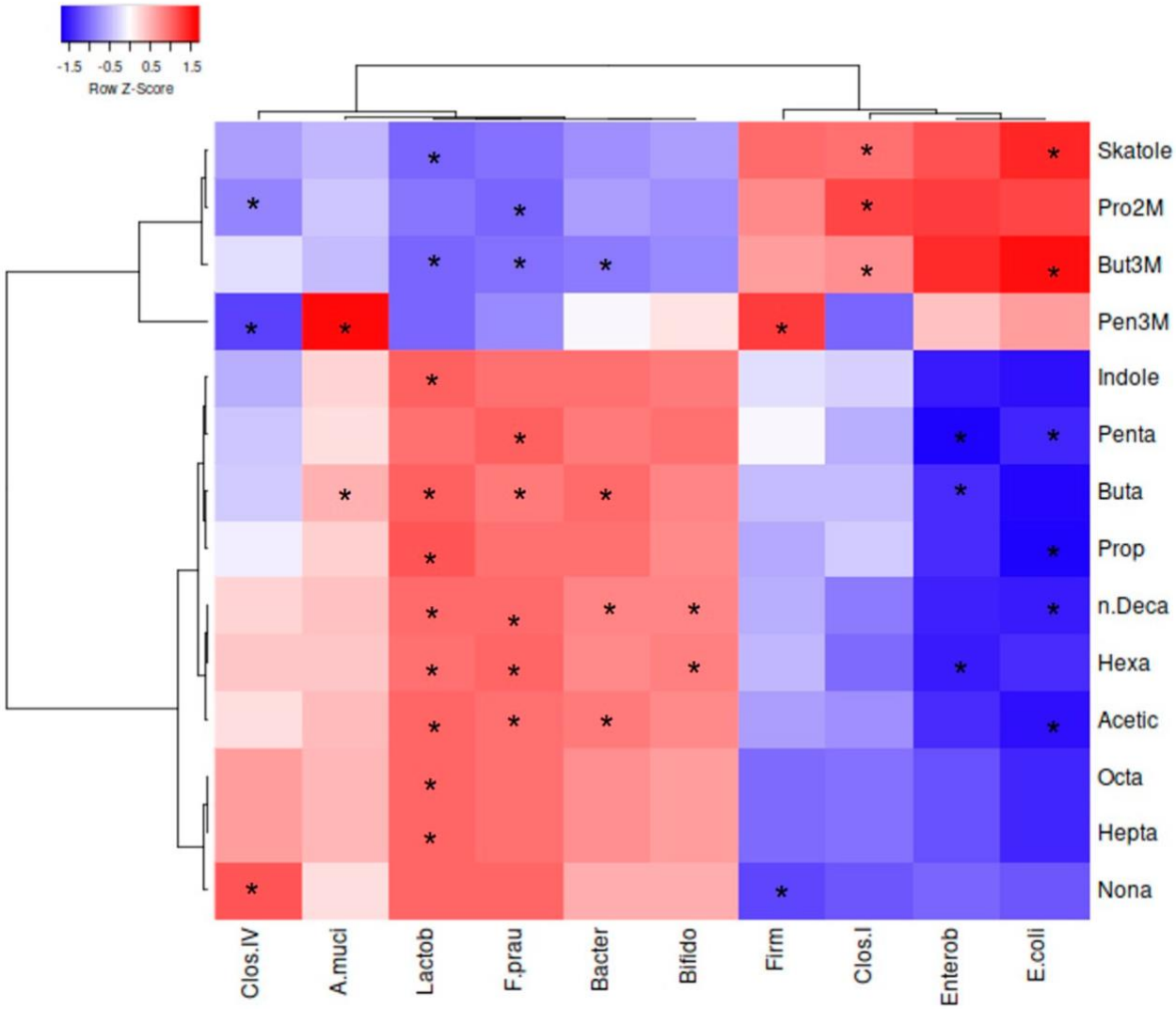

Figure 7. Spearman Rank Correlations among VOCs and microbial groups. *Clos.IV = Clostridium group IV*; *A.muci = Akkermansia muciniphila*; *Lactob = Lactobacillales*; *F.prau = Faecalibacterium prausnitzii*; *Bacter = Bacteroidetes*; *Bifido = Bifidobacteriaceae*; *Firm = Firmicutes*; *Clos.I = Clostridium group I*; *Enterob = Enterobacteriaceae*; *E. coli = Escherichia coli*; Pro2M = Propanoic acid, 2-methyl; But3M = Butanoic acid, 3-methyl; Pen3M = Pentanoic acid, 3-methyl; Penta =

Pentanoic acid; Buta = Butanoic acid; Prop = Propanoic acid; Acetic = Acetic acid; n.Deca = n-Decanoic acid; Hexa = Hexanoic acid; Octa = Octanoic acid; Hepta = Heptanoic acid; Nona = Nonanoic acid. Dendrograms are made by Pearson analysis with complete linkage. * Significance of correlations by Spearman Rank analysis ($p < 0.05$) (p values are reported in Figure S2).

7.5. Conclusions

During colonic fermentation, SCFAs or MCFAs derive from the degradation of the fiber components by resident colon microbiota. Their abundances represent good indicators of the modulatory effect of the fiber on the colon microbiota. Considering our outputs, it was possible to unveil the prebiotic potential of a new ingredient as FLS which was able to reproduce, but delayed the prebiotic capacity exerted by FOS.

Generally, this delay makes FLS almost unresponsive for the colon microbiota in the first 18 h of fermentations. Presumably, the EO terpenes and terpenoids present in FLS played an antimicrobial activity at the early and intermediate time points of fermentations implicating a slower production of beneficial or reduction of detrimental compounds. Interestingly, even if the high terpenic speciation in FLS initially slowed bacterial growth and generated a sleepy bacterial metabolism, the major toxic compounds derived from D-Limonene oxidation (4-acetyl-1-methylcyclohexene) was removed by the action of colon microbiota. Analogously, microbial changes such as the beneficial reduction of *Enterobacteriaceae* or the containment of *Clostridium* group I and *E. coli* were exerted at the end point in FLS. Even the indicator of eubiosis F/B reached similar baseline values at the end point after earlier slightly increases. Parallelly, the qPI value of FLS scored the top at the end point, while that of FOS at the intermediate time point. Although FLS needed a prolonged time of fermentation, it could be able to reach and even the prebiotic activity of FOS, resulting in a very promising novel prebiotic supplement. From a fast microbial turnover and high production of beneficial compounds is foreseen as a good characteristic of a prebiotic, but the capacity to slow microbial metabolism as well as to contribute to a more stable microbial yield and composition over time could be useful for those consumers that are more susceptible to physiological imbalances. In this way, FLS, after further research, could have the potential to target specific consumers, such as the elderly.

7.6. References

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7.7. Supplementary materials

Table S1. Baseline values of VOCs related to prebiotic activity in mg/kg.

VOCs	**Baseline (mg/kg)**		
Acetic acid	0.288	±	0.083
Propanoic acid	0.141	±	0.027
Butanoic acid	0.129	±	0.002
Pentanoic acid	0.123	±	0.003
Hexanoic acid	0.000	±	0.000
Heptanoic acid	0.000	±	0.000
Octanoic acid	0.000	±	0.000
Nonanoic acid	0.000	±	0.000
n-Decanoic acid	0.000	±	0.000
Pentanoic acid, 3-methyl	0.142	±	0.058
Butanoic acid, 3-methyl	0.245	±	0.024
Propanoic acid, 2-methyl	0.304	±	0.011
Indole	16.250	±	5.515
1H-Indole, 3-methyl	0.541	±	0.112

Table S2. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	**Target**	**Sequenza 3'-5'**	**Bp**	**Reference**
Eubacteria	V3-V4 16 S	Eub518-R: ATTACCGCGGCTGCTGG Eub338-R: ACTCCTACGGGAGGCAG	147	Lane *et al*, 1992
Bacteroidetes	V3-V4 16 S	Bact934F: GGARCATGTGGTTTAATT Bact1060R: AGCTGACGACAACCATG	250	Guo *et al*, 2008
Firmicutes	V3-V4 16 S	Firm934F: GGAGYATGTGGTTTAATT Eub338R: ACTCCTACGGGAGGCAG	300	Guo *et al*, 2008
Enterobacteriaceae	V3-V4 16 S	Enterobac-f: TGCCGTAACTTCGGGAG Enterobac-r: TCAAGGACCAGTGTTCAG	450	Bartosh *et al*, 2004
E. coli (total)	FtsZ	EcFtsZ-F: GGTATCCTGACCGTTGCT EcFtsZ-R: ATACCTCGGCCCAGAACT	250	Zhou *et al*, 1994

CASE STUDY 4: Prebiotic potential of fiber plus D-Limonene food supplement

Lactobacillales	V3-V4 16 S	F-Lac: GCAGCAGTAGGGAATCT R-Lac: GCATTYCACCGCTACACA	340	Walter *et al*, 2001
Bifidobacteriaceae	RecA	RecAf: CGTYTCBCAGCCGGAYA RecAr: CCARVGCRCCGGTCATC	220	Masco et al., 2006
Akm. muciniphila	V3-V4 16 S	AkM1: CAGCACGTGAAGGTGGG AkM2: CCTTGCGGTTGGCTTCA	327	Guo *et al*, 2016
Clostridium group I	V3-V4 16 S	ClosI-F: TACCHRAGGAGGAAGCCAC ClosI-R: GTTCTTCCTAATCTCTACGCAT	148	Bartosh *et al*, 2004
Clostridium group IV	V3-V4 16 S	ClosIV-f: TTAACACAATAAGTWATC ClosIV-r: ACCTTCCTCCGTTTTGTC	400	Goldberg *et al*, 2013

*Bp = base pair

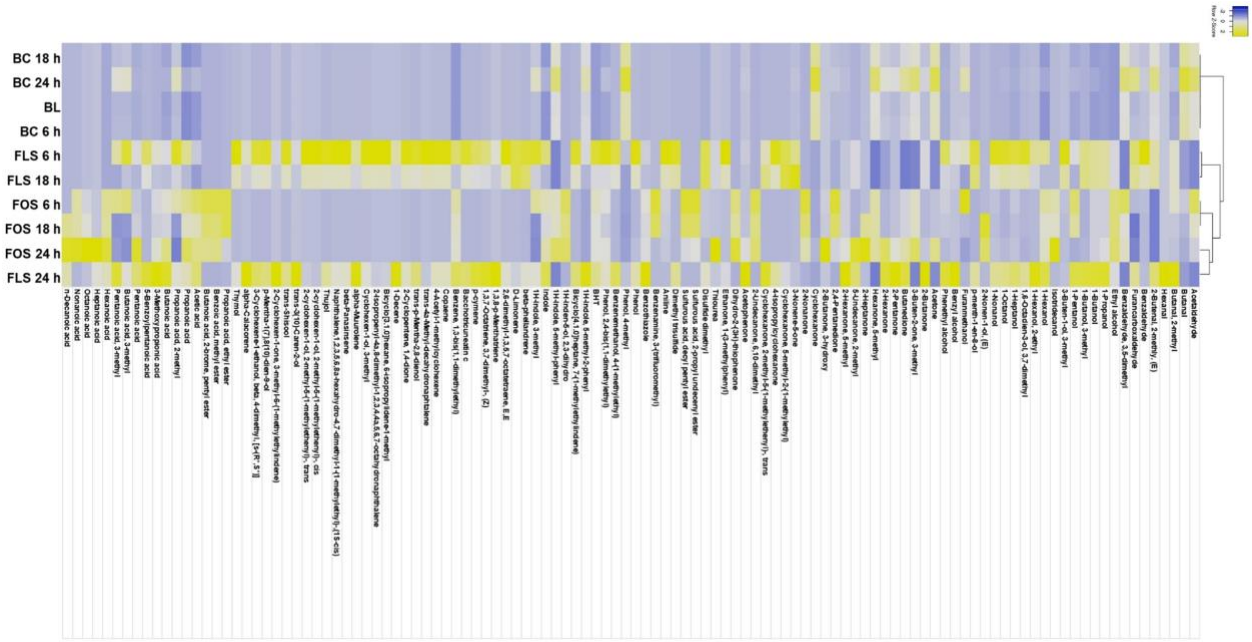

Figure S1. Quantification heatmap of total VOCs.

Table S3. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors significant amid the food matrices.

VOCs	FOS %	FLS %	P value
Acetaldehyde	100.00	0.00	0.021196
2-Butenal, 2-methly, (E)	0.00	100.00	0.004190
Benzaldehyde	3.74	96.26	0.046352
Furancarboxaldehyde	25.70	74.30	0.034243
Benzaldehyde, 3,5-dimethyl	100.00	0.00	0.004397
Ethyl alcohol	81.06	18.94	0.008725
3-Buten-1-ol, 3-methyl	3.74	96.26	0.049540
Isotridecanol	100.00	0.00	0.041860
1-Hexanol, 2-ethyl	4.92	95.08	0.022025
1,6-Octadien-3-ol, 3,7-dimethyl	5.00	95.00	0.000774
1-Octanol	4.91	95.09	0.000070
1-Nonanol	18.48	81.52	0.000010
p-Menth-1-en-8-ol	0.00	100.00	0.030519
3-Buten-2-one, 3-methyl	100.00	0.00	0.006583
Hexanone, 5-methyl	100.00	0.00	0.047791
2,4-Pentanedione	100.00	0.00	0.036135
4-Isopropylcyclohexanone	0.00	100.00	0.000550
p-Menthanone	0.00	100.00	0.002113
Acetophenone	21.30	78.70	0.027490
Ethanone, 1-(3-methylphenyl)	0.00	100.00	0.000015
Dimethyl trisulfide	0.00	100.00	0.000003
Benzenamine, 3-(trifluoromethyl)	100.00	0.00	0.040909
Phenol	6.47	93.53	0.000177
Benzenemethanol, 4-(1-methylethyl)	0.00	100.00	0.004301
1H-Inden-5-ol, 2,3-dihydro	100.00	0.00	0.001414
1H-Indole, 5-methyl-phenyl	100.00	0.00	0.009905

Table S4. MANOVA categorical descriptors for the volatilome, categorized for the time of fermentation. % of contribution of VOCs descriptors significant amid the time points.

VOC	6 h %	18 h %	24 h %	P value
Acetaldehyde	14.55	65.56	19.89	0.043432
Butanal, 2-methyl	11.28	45.71	43.01	0.015944
Hexanal	0.00	0.00	100.00	0.010567
Benzaldehyde	50.28	47.56	2.16	0.033290
1-Propanol	11.37	41.75	46.88	0.013369
1-Butanol	12.28	44.90	42.82	0.006751
1-Hexanol	17.10	37.00	45.90	0.012795
Benzyl alcohol	6.98	7.03	85.99	0.013642
2-Butanone	6.22	22.78	71.00	0.042579
Butanedione	4.34	23.55	72.11	0.003344
2-Pentanone	6.23	25.34	68.43	0.016105
2-Hexanone	1.20	6.25	92.55	0.005513
2-Butanone, 3-hydroxy	0.87	2.80	96.33	0.010938
p-Menthanone	8.96	45.72	45.32	0.043980
Acetophenone	1.73	2.08	96.20	0.011561
Thiourea	1.77	6.15	92.08	0.011358
Benzothiazole	8.10	18.83	73.07	0.007840
Phenol, 4-methyl	20.89	29.00	50.11	0.046953
Butylated hydroxytoluene	23.15	34.70	42.14	0.043657

Figure S2. Significance of Spearman rank correlations.

Variable	Spearman Rank Order Correlations (Spreadsheet4) MD pairwise deleted Marked correlations are significant at p <.05000 Firmicutes	Bacteroidetes	Lactobacillales	Bifidobacteriaceae	Enterobacteriaceae	Clostridium group I	Clostridium group IV	A muciniphila	E. coli	F. prausnitziii
Acetic acid	-0,235396	0,828571	0,942857	0,771429	-0,753702	-0,314286	0,371429	0,542857	-0,88571	0,885714
Propanoic acid	-0,235396	0,771429	0,885714	0,657143	-0,753702	-0,085714	0,085714	0,371429	-0,94286	0,771429
Butanoic acid	-0,147122	0,885714	0,942857	0,771429	-0,811679	-0,142857	-0,085714	0,542857	-1,00000	0,828571
Pentanoic acid	0,088273	0,714286	0,771429	0,771429	-0,985611	-0,257143	-0,142857	0,257143	-0,82857	0,828571
Hexanoic acid	-0,117698	0,771429	0,885714	0,828571	-0,840668	-0,485714	0,485714	0,485714	-0,77143	0,942857
Heptanoic acid	-0,441367	0,657143	0,828571	0,600000	-0,550782	-0,428571	0,600000	0,485714	-0,71429	0,771429
Octanoic acid	-0,441367	0,657143	0,828571	0,600000	-0,550782	-0,428571	0,600000	0,485714	-0,71429	0,771429
Nonanoic acid	-0,562775	0,394665	0,637536	0,394665	-0,462031	-0,516100	0,698253	0,212512	-0,51610	0,637536
n-Decanoic acid	-0,179124	0,811679	0,927634	0,811679	-0,808824	-0,405840	0,434828	0,521794	-0,84067	0,927634
Pentanoic acid, 3-methyl	0,411943	0,085714	-0,142857	0,142857	0,202920	-0,142857	-0,200000	0,485714	0,25714	-0,085714
Butanoic acid, 3-methyl	0,235396	-0,828571	-0,942857	-0,771429	0,753702	0,314286	-0,371429	-0,542857	0,88571	-0,885714
Propanoic acid, 2-methyl	0,323669	-0,600000	-0,771429	-0,657143	0,637748	0,600000	-0,714286	-0,428571	0,60000	-0,828571
Indole	-0,029424	0,771429	0,828571	0,714286	-0,898645	-0,085714	-0,257143	0,314286	-0,94286	0,771429
1H-Indole, 3-methyl	0,441367	-0,657143	-0,828571	-0,600000	0,550782	0,428571	-0,600000	-0,485714	0,71429	-0,771429

8. CASE STUDY 5: Beneficial metabolic transformations and prebiotic potential of hemp bran and its alcalase hydrolysate, after colonic fermentation in a gut model

(Under minor revisions for Scientific Reports)

8.1. Introduction

Hemp seed is a powerhouse of nutrients and a mine of bioactives, bearing some exceptional issues as: being sugarless, low in glycemic, gluten free, rich in balanced ratio of PUFA, neuro strengthener, cardiovascular protection, etc (Lin et al., 2021). A principal criterium of OneHealth philosophy is sustainability of the food industry, that should be guided to the reduction of wastes and by the exploitation of byproducts. Considering this scenario, we recently have set attention on Hemp seed bran (HB), which is an unexplored byproduct of the industrial processing of hemp seed. A better exploitation of the industrial food chain production is a need, and the valorization of HB, which is treated as a byproduct and mainly discarded, is our main focus. Indeed, we previously characterized HB for its ability to foster the growth of beneficial bacteria and to exhibit potent prebiotic potential depending on its level of processing (Setti et al., 2020; Nissen et al., 2021). New functional ingredients for bakery foods, thereby increasing their nutritional value were also obtained (Nissen et al., 2021).

Considering the use of HB as an innovative and low-cost source to formulate healthier and value-added foods, its impact on human colon microbiota and an exhaustive explanation and comprehension of its prebiotic potential are situations to investigate on. In fact, the latest definition of prebiotics[4] indicates that there are other compounds than oligosaccharides that can get the claim, such as polyphenols and terpenes, of which HB is rich (Nissen et al., 2021). By definition, prebiotics are degraded by colon microbes and influence the whole microbiota; directly feeding the commensals group and fostering probiotics towards eubiosis and consequently to host health (Gibson et al., 2017). The action of a prebiotic is also directed to the limitation of opportunistic bacterial groups that produce toxic catabolites, such as phenols and some indoles (Wang et al., 2020).

In this state, HB and its enzymatic hydrolysates could play an important role, because, as we have previously demonstrated in *in vitro* studies with type strains bacteria, other than carbohydrate

compounds, HB brings many bioactives, *e.g. p*-Cymene, Caryophillene, 4-Terpineol, Acetophenone, Myrtenal (Nissen et al., 2021).

In vitro gut models are considered a proper solution to study the impact of dietary beneficial compounds on human gut microbiota (Nissen et al., 2020; Perez-Burillo et al., 2021). Throughout the assessment of microbiomics (ecological diversities and the shift of the microbiota), metabolomics (volatilome profiling), and inter-omics (correlations of the previous two) it is possible to unveil the cause and effects of the fiber's functionalities. In this context, *in vitro* colon fermentation was simulated with the aid of a human colon model, namely MICODE (Multi-unit in vitro Colon Model) a versatile colon model (Nissen et al., 2021a; Nissen et al., 2021b) to specifically study prebiotic potential of HB and of alcalase-treated HB protein isolate (HBPA), by simulating the distal part of the human large intestine. HBPA was previously characterized for its antioxidant and antiypertensive properties (Setti et al., 2020), also related to the presence of bioactive peptides originated from protein hydrolysis (Samaei et al., 2021).

We used MICODE with fecal donations from three healthy donors for a short-term colonic fermentation protocol (24 h) of HB and HBPA in comparison to prebiotics (FOS) as positive control and to a blank control. Aiming at the understanding of the potential health benefits of HB alcalase hydrolysates, an inter-omic approach coupling microbial genomics (qPCR and Illumina sequencing) and metabolomics (SPME GC-MS) was adopted, focusing on ecological indicators such as: i) microbial biodiversity, ii) microbial eubiosis, iii) prebiotic index, iv) production of prebiotic compounds, such as SCFAs and MCFAs, v) reduction of detrimental compounds, such as phenols and p-Cresol, vi) presence of bioactive volatiles, such as Borneol and Acetophenone, and vii) shift in those bacterial taxa specialized in fiber degradation or in proteolytic fermentations.

8.2.Materials and methods

8.2.1. Fecal donors

The fecal samples used for this study were obtained from three lean healthy individuals that respected the inclusion criteria as previously reported (Connoly et al, 2012; Koutsos et al., 2017; Oba et al., 2020). Fecal samples were collected, and processed as previously described (Nissen et al., 2021a; Nissen et al., 2021b).

8.2.2. Materials

Chemicals, solvents, and enzymes for batch culture fermentation were of the highest analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), and

Carlo Erba Reagents (CEDEX, Val de Reuil, FR), unless otherwise stated. Reagents for molecular biology and kits for DNA extraction or purifications were purchased from Thermo Fisher Scientific (USA).

8.2.3. Experimental sample and controls

Experimental HB was previously prepared and characterized (Setti et al., 2020; Nissen et al., 2021). HB, the byproduct remaining after mechanical pressing of hemp seeds and subsequent grinding and sieving, was supplied by a local company (Hemp Positive World, Cesena, Italy). The original hemp variety was Futura 75. To prepare HB samples for fermentation, 50 g of bran were resuspended in 300 mL of distilled water, sterilized (121 °C and 100 kPa for 20 min) (Vapor Matic 770, ASAL Srl, Milan, Italy) in 500 mL Corning - Pyrex bottles (Corning. NY, USA), aseptically poured on sterilized metal vessels, and stored at -80 °C.

HBPA was produced by enzymatic digestion of chemically extracted HB protein isolate, as described by Setti et al. (2020), extracting protein from HB following published procedure[15]. Briefly, the protein isolate was dissolved in deionized water (1:8, w/v) and hydrolyzed for 2h with Alcalase (2%, v/v) at 50°C and pH 8.0. The enzyme was then heat inactivated at 85°C for 15 min. After cooling down to room temperature, the solution was centrifuged at 14000 x *g* for 10 min, and the supernatant was collected and stored at -80°C.

Both HB and HPBA solutions were freeze dried with a Savant freeze-dryer Lyolab 3000 apparatus (Thermo Fisher Scientific, USA), and the powder was used to test the prebiotic potential.

8.2.4. Fecal batch-culture fermentation and samples collection

Colonic fermentations were conducted in independent vessels on 1 % (w/v) of HB, on 1 % (w/v) of HBPA, on 1 % (w/v) of fructo-oligosaccharides (FOS) from chicory (positive control), and on a blank control (BC) (negative control), using MICODE. pH was adjusted to 6.75 and kept throughtout the experiment to mimic the conditions located in the distal region of the human large intestine. For this work, the BL was set after 2.55 ± 0.11 h. Samples of the different time points were used for SPME-GC-MS and qPCR. Technical replicas of analyses were conducted in triplicates from two independent biological experiments. The freeze-dried samples were directly fermented in the colon with no gastric phase digestion, as the nature of prebiotic is to reach the colon to feed the microbiota without being affected by host's enzymes (Sanders et al., 2019).

8.2.5. Pipeline of experimental activities

Parallel and independent vessels for FOS, HB, HBPA, and BC were run for 24 h after the adaptation of the fecal inoculum, defined as the baseline (BL). The entire experiment consisted of 32 cases (n = 32), including 4 theses (FOS, HB, HBPA, and BC) and 4 time points (BL, 6 h, 18 h, and 24 h) in duplicate. Samples of the different time points were used for qPCR and SPME GC-MS analyses. After sterile sampling of 5 mL of bioreactor contents, samples were centrifuged at 16000 × g for 7 min to separate the pellets and the supernatants, which were used for bacterial DNA extraction and SPME-GC-MS analysis, respectively. Specifically, microbial DNA extraction was conducted just after sampling so as not to reduce *Firmicutes* content. Sampling from DNA samples and SPME-GC-MS samples were then stored at -80 °C. Technical replicas of analyses were conducted in duplicate for SPME GC-MS (n = 64) and in triplicate for qPCR (n = 96), both from two independent experiments.

8.2.6. Microbiota related analyses

8.2.6.1. *DNA extraction, amplification, and sequencing*

DNA was extracted from the fecal samples (from donors and the pool) and from the MICODE effluates at each time points (BL, 6 h, 18 h, and 24 h) using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK). Samples from the feces, the BL, and the end point were used for MiSeq sequencing (Illumina Inc, San Diego, CA, USA), while samples from the BL and other time points were used for quantitative PCR (qPCR) analyses. Considering the MiSeq approach, bacterial diversity was obtained by the library preparation and sequencing of the 16S rRNA gene. The following two amplification steps were performed: an initial PCR amplification using 16S locus-specific PCR primers (16S-341F 5′-CCTACGGGNGGCWGCAG-3′ and 16S-805R 5′-GACTACHVGGGTATCTAATCC-3′) and a subsequent amplification integrating relevant flow-cell-binding domains (5′-TCGTCG GCAGCGTCAGATGTGTATAAGAGACAG-3′ for the forward primer and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ for the reverse overhang), and lastly unique indices selected among those available Nextera XT Index Kits were combined according to manufacturer's instructions (Illumina Inc, USA). Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, USA). In addition, libraries were quality-tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent technologies, Santa Clara, CA, USA). Libraries

were sequenced in a MiSeq (Illumina Inc, USA) in the paired end with 300-bp read length. Sequencing was conducted by IGA Technology Service Srl (Udine, Italy).

8.2.6.2. Sequence data analysis

Reads were de-multiplexed based on Illumina indexing system. Sequences were analyzed using QIIME 1.5.018. After filtering based on read quality and length (minimum quality = 25 and minimum length = 200), Operational Taxonomic Units (OTUs) defined by a 97% of similarity were picked using the Uclust v1.2.22 q method (Edgar et al., 2010) and the representative sequences were submitted to the RDP classifier20 to obtain the taxonomy assignment and the relative abundance of each OTU using the Greengenes 16S rRNA gene database (McDonald et al., 2011). Alpha- and beta-diversity analyses were performed using QIIME 1.5.0.

8.2.6.3. *Enumeration of bacterial group for the qPI (Quantitative Prebiotic Index)*

Changes in *Eubacteria kingdom, Lactobacillales order, Bifidobacteriaceae, Enterobacteriaceae families, and Clostridium group I* were also assessed by qPCR and SYBR Green I chemistry (Nissen et al., 2021; Tanner et al., 2014; Tsitko et al., 2019), targeting small fragments of monocopies, or multicopy genes by degenerated or specific primer pairs, previously amplified by high-fidelity DNA polymerase (Invitrogen Platinum SuperFi II DNA Polymerase, Thermo Fischer Scientific, USA). Extraction of bacterial DNA was obtained with Pure Link Microbiome DNA Purification Kit (Invitrogen, USA). Genetic standards were prepared from relative PCR amplicons of the target bacterial species, using GeneJet Genomic DNA purification kit (Thermo Fisher Scientific, USA), as described previously24,8. For each of the targets, general qPCR reactions were set as follows: a holding stage at 98 °C for 6 min, and a cycling stage made of 95 °C for 20 sec and 60 °C for 60 sec, repeated for 45 times, followed by melting curve analysis. Quantifications were made by a RotorGene 6000 (Qiagen, Hilden, Germany) with a five-point standard of the given amplicon, separately. Reactions were prepared with 1 ng of DNA, 2x Power-Up SYBR Green (Thermo Fisher Scientific, USA) and 250 nM of each primer (Eurofins Genomics, Ebersberg, Germany). Details of primer pairs for PCR and qPCR are supplied as Table S1. All results were expressed as mean values obtained from triplicates from two independent experiments.

The Prebiotic Index was revised from the original equation elaborated by Palframan et al. (2003) introducing substitution on bacterial taxa, the molecular approach based on quicker qPCR, data normalization, sextuplicate values, and significative differences9. Analogously to the original

method, we employed an equation based on quantification values expressed as Log10 cell/mL, and similar conditions applied in fermentation (24 h controlled batch with 1% w/v of prebiotic fiber). So far, the new equation for the Prebiotic Index is based on qPCR data (qPCR Prebiotic Index - qPI) as follows: qPI = (*Bifidobacteriaceae/Eubacteria)* – *(Enterobacteriaceae/Eubacteria)* + *(Lactobacillales/Eubacteria)* – *(Clostridium group I/Eubacteria).*

8.2.7. Volatilome analysis

Volatile organic compounds (VOCs) evaluation was performed on a Gas Chromatograph (7890A , Agilent, Santa Clara, CA, USA) Mass Spectrometer (5975, Agilent, USA) operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME (Solid Phase Micro-Extraction) GC-MS protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Nissen et al., 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Preconditioning, absorption, desorption of SPME–GC-MS analysis, and all data processing were carried out according to literature (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021). Molecules Identification was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). The VOCs were then sorted and super-normalized for respective chemical class, i.e., organic acids, alcohols, and other VOCs (Nissen et al., 2021a). In samples at BL the main microbial metabolites related to fermentation of foods were absolutely quantified in mM by SPME GC-MS and an internal standard (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021a; Di Cagno et al., 2011; Casciano et al., 2021).

8.2.8. Data processing and statistical analysis

For the volatilome, one-way ANOVA ($p < 0.05$) was used to determine significant VOCs among the dataset, which included 8000 interactions generated between 125 dependent variables (VOCs) and 64 independent variables (2 technical and 2 experimental replicas of 4 different fermentation treatments; HBPA, HB, FOS, and blank control, and 4 different time points; Baseline, 6, 18, and 24 h). The significant VOCs (n = 91) represented the total volatilome of the experiments and was reported as a quantification heatmap (Figure S2). Then, from this dataset the VOCs were divided in three groups, and analyzed differently: i) the prebiotic related VOCs (preVOCs); ii) the detrimental VOCs and iii) the remaining volatilome. The analyses conducted were Principal Component Analysis (PCA) to distribute the results on a plane; Multivariate ANOVA (MANOVA) to address specific

contributes by categorical predictors; Student's t-test to compare a sample to another within the same variable.

For the microbiota, after ANOVA for group comparison (the baseline versus the end point), the significant variables ($p < 0.05$) were selected and the shifts in abundance were calculated as $Log_2(F/C)$. Then, *post hoc* Tukey HSD test ($p < 0.05$) was performed on the raw data to define differences among treatments. The microbiota at the endpoint was analyzed as a pool of DNA of the biological replicas for each case, while at the baseline as a pool of the four cases. For the qPI values a dataset of 5 dependent variables (bacterial taxa) and 96 independent variables (3 technical and 2 experimental replicas of 4 different fermentation treatments, and 4 different time points, Baseline, 6, 18, and 24 h) was studied for statistical differences among time points and treatments by ANOVA ($p > 0.05$) and *post hoc* Tukey HSD test ($p < 0.05$).

To address specific correlations among bacteria and molecules (preVOCs) and explain the prebiotic potential of HBPA, two independent HBPA datasets were merged and computed by Spearman Rank analysis and visualized with a two-way joining heatmap including Pearson dendrograms with complete linkage. The baselines of values for the volatilome and for the microbiota were that obtained sampling just after adaptation of the microbiota to the bioreactor condition[9]. Normalization of datasets was performed with the mean centering method. Statistics and graphics were made with Statistica v.8.0 (Tibco, Palo Alto, CA, USA), but two ways joining heatmap graphic was performed with Expression tool on www.heatmapper.ca.

8.3.Results and discussion

8.3.1. Quality controls for the validation of MICODE protocol

To validate the MICODE experimental approach in the version of fecal batch of the human proximal colon, we chose to monitor and check some parameters as quality controls (QC) related to metabolites and microbes at the end of fermentations, and in comparison, to the baseline. QCs adopted were; i) the *Firmicutes/Bacteroidetes* ratio (F/B), which is related to health and disease (Koliada et al., 2017), was maintained at a low level, confirming the capacity to simulate a healthy in vivo condition for 24 h. ii) The presence of *Archea* (*e.g.*, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*), which are pretty sensible to oxygen content (Samuel et al., 2007), was retained from the baseline to the end point in each vessel and repetition, indicating that the environmental conditions were strictly maintained. iii) Good's rarity index of alpha biodiversity remained similar during time of fermentation ($p > 0.05$), indicating enough support to the growth of rare species. iv) Observed OTUs richness index scored approximately 400 OTUs at the end point. v) The paradigm of prebiotics was

confirmed when the positive control (FOS) was applied on MICODE; high probiotic and SCFAs increases and limitation of enteropathogens. vi) Each GC/MS analysis had quantified some stool-related compounds (Urea, 1-Propanol, and Butylated hydroxy toluene), that ranged across the complete chromatogram and were adsorbed at the same retention times.

8.3.2. Changes in bacterial alpha and beta diversities

The microbiota diversity indices were analyzed to study the impact of HPBA on microbial population, to assess population's stability during fermentation, and to compare its microbiota to that of other bioreactors (Figure S1). The baseline of value was compared to the endpoints of fermentation of different treatments. It is undisputable that a part of the effect of reduction in richness (Observed OTUs) was derived by the passage from *in vivo* to *in vitro* condition, but the focus must be set on the different trend that other alpha diversity indices had. For example, abundance (Chao 1) for HBPA was significantly higher at the end of fermentation ($p < 0.05$), while a not significant reduction was seen for HB or FOS. Surges in evenness (Shannon) were seen for HB ($p > 0.05$) and HPBA ($p < 0.05$), but no changes were seen in dominance (Simpson) ($p > 0.05$), while oppositely, FOS decreased in evenness ($p > 0.05$) and raised in dominance ($p < 0.05$). This output indicates a different performance of HPBA or minorly HB in respect to FOS and is well explained by the trend of dominance that tells that for FOS some taxa overcame others, reducing the uniform distribution of bacterial groups in the microbiota. This effect was already observed, and could be justified by the ability of FOS to foster *Bifidobacteriaceae* and make them dominant over the microbiota (Nissen et al., 2021; Tsitko et al., 2019). HBPA and minorly HB instead had an effect with a wider range of bioactivity on more bacterial targets; that higher biodiversity could be seen as an added value on its prebiotic potential.

When the bacterial diversity between samples (beta diversity) was examined with Bray–Curtis analysis, the fecal samples was set distant to the BL, and the BL distant to the end point cases, as demonstrated by principal coordinate analysis (PCoA) based on an unweighted (qualitative) phylogenetic UniFrac distance matrix. This feature confirms that shifts occurred during the experiments. Additionally, the four cases at the end point were relatively distant one to each other. This feature confirms that different shifts occurred from the BL on. So far, the study of biodiversity indicated the ability to keep an eubiosis conditions by fermentations of both the hempseed bran samples, with generally a higher capacity of HBPA in respect to HB. Considering that HBPA should have a higher availability of shorter fiber chains and more unbound saccharides due to the action of alcalase treatment (Setti et al., 2020), that result could indicate that HBPA is generally more appetible for colonic fermentation than HB.

8.3.3. Changes in taxa abundances at the phylum and species level

The total sequence reads used in this study were classified into eight phyla and one unassigned (Table S1). In any tested sample, the core microbiota was represented by five taxa: three with a relative abundance higher than 10% (*Firmicutes*, *Bacteroidetes*, and *Actinobacteria*) and two lower than 3% (*Proteobacteria* and *Verrucomicrobia*). Anyhow, just *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* underwent significative changes in comparison to the baseline ($p < 0.05$).

As a general parameter for microbiota eubiosis we chose the famous ratio *Firmicutes*/*Bacteroidetes* (F/B), and we considered the differences from the baseline to the end point. Within this ratio a value over two is usually referred to microbiota dysbiosis (Koliada et al., 2017; Zhou et al., 2017). The fecal samples at the baseline had a F/B of 1.66 and this eubiosis condition was maintained by HBPA (1.55), by HB (1.62) and FOS (0.73), although significantly just for the latter ($p > 0.05$). These results indicate that during the time of fermentation, HB and HBPA did not perturb the colon core microbiota of healthy donors, but was able to provide a substrate that meet the energetic expenditure of the microbiota, keeping an eubiosis condition.

A dataset of significant OTUs changes relative to the family level is reported in Table S6. Anyhow, we focused the discussion on results obtained at the specie taxonomic level, where 113 OTUs were constructed and assigned to microbial taxa (cutoffs 0.001%). Of these, 113 were identified at the baseline, while 106, 102, 96, and 100 were identified at the endpoint of fermentations with HPBA, HB, FOS, and the blank control, respectively. Then a dataset of 41 microbial OTUs was selected and tested for ANOVA group comparison in respect to the baseline ($p > 0.05$). Among these, 31 variables were significant and their Log_2 fold changes in respect to the baseline were compared by post-hoc test (Table 1). The 41 OTUs selected were those that recorded shifts after fermentation and that from literature are susceptible to the effect of prebiotic or fiber substrates. We have included even three OTUs of *Archea* relative to QC of the experiments (previously discussed in *5.3.1*).

The first group of OTUs included beneficial or commensal bacteria that usually respond to prebiotics. In this group, three *Bifidobacterium* were picked showing increases on the substrates and reduction on the blank control. HB and HBPA fostered *Bif. bifidum*, but just the latter did it significantly, making this taxon grew up to the 3.30% of relative abundance ($p < 0.05$). Besides, FOS fostered even *Bif. adolescentis* ($p < 0.05$). Among *Bacteroides*, five OTUs were chosen and except *B. fragilis* were all significant ($p < 0.05$). *B. thetaiotaomicron* and *B. uniformis* were the most abundant in HBPA, HB, and FOS bioreactors at the endpoint, the first recorded top shift for HBPA reaching 7.88% of total abundance. *Parabacteroides distasonis* was found rich and significantly increased after

fermentation with HBPA, HB, and FOS ($p < 0.05$), but not in the blank control ($p > 0.05$). From the class of *Lactobacillales*, significant shifts ($p < 0.05$) were seen for two *Enterococcus* and two *Lactobacillus* OTUs, that augmented with the substrates and decreased in the blank control. Interestingly, while *En. durans* was largely fostered by both HBPA, HB, and FOS, *En. faecalis* just by HBPA and HB and reduced by FOS. *Lactobacillus mucosae* and *Lb. plantarum* were represented in very low amounts at the baseline and were intensively fostered by both substrates. For example, the first reached the top quantity of 0.06% with HBPA, while the second that of 0.24% with FOS. *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* were more abundant after substrates fermentation and less in the blank control, although not all significantly ($p < 0.05$).

From our results, even at the depth of the species level, it was possible to highlight the prebiotic potential of HB and on larger extend of HBPA that, similarly to FOS, fostered several taxa of beneficial bacteria. In particular, the surges in these taxa were relative to: i) three species of health associated and SCFAs-producer *Bifidobacterium* (Gibson et al., 2017); ii) MCFAs- and sphingolipids-producer *B. thetaiotaomicron*, succinate-producer *P. distasonis* (Oba et al., 2020; Hiippala et al., 2020), and iii) competitive excluders *Lactobacillales*, as *Lb. plantarum* and *E. durans* (Callaway et al., 2008; Soltani et al., 2021*)*. Moreover, HBPA showed to be able to foster beneficial SCFAs-producer *F. prausnitzii*[5] and fiber-degrading *B. caccae* (Nakajima et al., 2020). In comparison to HB, the better performance obtained by HBPA are due the action of alcalase that gives a product with a higher rate proteins/peptides with MW around or lower than 15 kDa, and an increased percentage of soluble proteins (10%) (Setti et al., 2020). It is reported, for example that *Lactobacillaceae* likes peptides more than proteins, and prefers to ferment low molecular weight peptides than proteins (Al-Tamimi et al., 2006).

A second list of bacterial taxa, that were subject to changes in abundance at the endpoint, was that of opportunistic species. *Bilophila wadsworthia*, *Desulfovibrio* (pathobiontic, highly proteolytic and sulphate producers) and *Escherichia albertii,* (close relative to pathogenic species) were reduced by HBPA, HB, and FOS while increased in the blank control ($p < 0.05$). In particular HPBA performed better than HB and FOS in the containment of *Bil. wadsworthia* and *Desulfovibrio.* In details, considering these three taxa, HBPA was stronger than HB but with no significant differences in the reductions ($p > 0.05$), except for that relative to *B. wadsworthia* ($p < 0.05$). The ability to counteract opportunistic and enteropathogenic microbes is an essential feature of a prebiotic compounds. Particularly, these species are involved in dysbiosis of the microbiota and pathogenesis (Wang et al., 2007; Denamur et al., 2021) and were reduced in a similar study on prebiotics and vegetal fibers (Wang et al., 2020). Thus, HBPA had superior performances than HB in limiting the development of

opportunistic microbes. This evidence may contribute to explain the beneficial effects of hydrolyzed proteins. Indeed, the modulation of gut microbiota usually results from unabsorbed sugars, resistant starch, and fibers, but indigestible proteins and bioactive peptides have been proven beneficial too, such as hydrolysed proteins from soy (Ashaolu et al., 2019). Additionally, this feature could in part attributable to the increased release of peptides with higher antioxidant capacity when HB underwent the alcalase treatment, as well as an higher content of bioactive peptides. In fact, Setti et al. [2] found that HBPA in comparison to HB have a 7 times stronger activity on ABTS assay and a 10 times stronger activity on FRAP assays. Additionally, Samaei et al. (2021) identified on HBPA 47 bioactive peptides, that for the most are short sequences of a few amino acids, potentially resistant to the gastrointestinal conditions.

The third list regards to those taxa that usually respond to vegetal fibers. The performances of HBPA that deserve merit of notion are the significant reduction of *Ruminococcus gnavus* and *R. torques,* as well as that of *Colinsella aerofaciens* and *Eggerthella lenta*, similarly to FOS ($p < 0.05$). The two *Ruminococcus* are culprits of dysbiosis associated to intestinal syndromes and are effective responders to fiber diet regime (Zhu et al., 2020). In contrast to FOS, HPBA and minorly HB were able to increase the quantity of *Oscillospira* and *Sutterella*. For HBPA the surges were significant reaching 11.90%, and 1.94% of relative abundance, respectively ($p < 0.05$). Despite the role in gut microbiota of *Oscillospira* remains enigmatic, as a member of *Ruminococcaceae* should be implicated in fiber degradation (Konikoff et al., 2016) and could explain the reduced abundance observed in HPBA for same family member *F. prausnitzii*. *Sutterella*, in the past indicated as an opportunistic species has been recently reconsidered for its ability to degrade plant-based pectins and similar compounds in *in vitro* systems likely the MICODE (Barnett et al., 2021).

Table 1. Abundances (% ± S.D.) and changes in phylum taxa (Log_2 F/C) after 24 h *in vitro* fecal batch culture fermentations from healthy donors and administrated with HBPA, HB, and FOS as the substrates, and also including a blank control.

	% Relative abund.	**Log_2(F/C) changes at the end points (24 h)**				***p* value***
Taxon	**Baseline Mean**	**HBPA**	**FOS**	**HB**	**Blank**	
Quality controls						
Archaea;Other	0.001 ± 0.001[b]	3.27[a]	2.07[ab]	3.07[a]	n.d.	0.03749
Methanobrevibacter;s__smithii	0.486 ± 0.411	-4.27	-5.88	-2.27	-9.10	0.45636
Methanosphaera;s__stadtmanae	0.003 ± 0.004	0.05	-1.14	0.12	-1.08	0.92717

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Prebiotic sensitive (beneficial and commensal taxa)						
Bifidobacterium;s__adolescentis	4.414 ± 1.743[b]	0.38[b]	1.08[a]	0.21[b]	-0.27[b]	0.03883
Bifidobacterium;s__bifidum	0.974 ± 0.177[b]	1.76[a]	1.67[a]	1.16[a]	-0.25[b]	0.00132
Bifidobacterium;s__longum	2.744 ± 0.544	0.45	0.55	0.22	-0.04	0.15503
Bacteroides;s__acidifaciens	0.115 ± 0.009[b]	1.07[a]	1.39[a]	0.29[b]	-0.27[b]	0.00052
Bacteroides;s__caccae	0.713 ± 0.086[b]	1.12[a]	1.46[a]	0.22[b]	-0.79[b]	0.00113
Bacteroides;s__fragilis	0.238 ± 0.188	2.07	1.51	1.01	-1.85	0.05038
Bacteroides;s__thetaiotaomicron	0.393 ± 0.093[c]	4.32[a]	2.90[b]	3.30[a]	0.78[c]	0.00001
Bacteroides;s__uniformis	3.583 ± 0.301[c]	1.39[b]	2.92[a]	0.39[c]	-2.84[d]	0.00001
Parabacteroides;s__distasonis	0.666 ± 0.270[b]	2.32[a]	2.95[a]	1.84[a]	0.49[b]	0.00109
Enterococcus;s__durans	0.400 ± 0.670[b]	3.55[a]	4.36[a]	3.01[a]	-2.10[b]	0.00305
Enterococcus;s__faecalis	0.005 ± 0.007[b]	6.77[a]	-1.06[b]	4.21[a]	0.81[b]	0.00001
Lactobacillus;s__casei	0.015 ± 0.022	1.78	0.77	1.05	0.01	0.35888
Lactobacillus;s__manihotivorans	0.021 ± 0.031	1.74	1.30	0.84	0.57	0.37442
Lactobacillus;s__mucosae	0.003 ± 0.002[b]	4.48[a]	4.50[a]	3.35[a]	-0.05[b]	0.00010
Lactobacillus;s__plantarum	0.001 ± 0.000[c]	5.00[b]	7.54[a]	4.02[b]	-0.03[d]	0.00001
Streptococcus;s__thermophilus	0.533 ± 0.359	-0.65	0.43	-0.45	-2.06	0.56392
Roseburia;s__faecis	0.096 ± 0.035	-2.00	-2.61	-1.80	-4.55	0.09065
Faecalibacterium;s__prausnitzii	1.734 ± 0.770[a]	0.36[a]	0.31[a]	0.12[a]	-3.22[b]	0.01857
Akkermansia;s__muciniphila	0.903 ± 0.122[a]	0.30[a]	0.55[a]	0.19[a]	-4.82[b]	0.00869
Prebiotic sensitive (opportunistic taxa)						
Streptococcus;s__pseudopneumoniae	0.080 ± 0.073	-5.73	-4.93	-5.02	-0.54	0.51847
Bilophila;s__wadsworthia	0.149 ± 0.019[b]	-3.04[c]	-2.12[c]	-2.04[c]	2.67[a]	0.00006
Citrobacter;s__freundii	0.051 ± 0.030	-0.45	-5.29	-0.66	1.67	0.05761
Escherichia;s__albertii	0.064 ± 0.042[b]	-0.19[b]	-0.59[b]	-0.17[b]	3.39[a]	0.00202
Desulfovibrio;s__	0.395 ± 0.117[a]	-2.07[b]	-1.59[b]	-1.66[b]	0.06[a]	0.04574
Peptostreptococcaceae;g__Clostridium;s__	0.062 ± 0.053	-0.41	-3.24	-0.56	1.42	0.23301
Vegetal Fiber sensitive (positive)						
Blautia;s__	6.422 ± 1.734	-6.20	-3.78	-4.13	-2.40	0.05643

Blautia;s__obeum	0.977 ± 0.205[a]	-4.88[b]	-4.15[b]	-4.10[b]	-0.81[ab]	0.02860
Ruminococcus;s__gnavus	2.203 ± 0.720[a]	-5.38[b]	-3.11[b]	-4.11[b]	-0.28[a]	0.04652
Ruminococcus;s__torques	0.694 ± 0.607	-7.26	-9.04	-6.06	-0.66	0.47420
Tepidibacter;s__	1.886 ± 0.445	-0.93	-2.17	-0.15	0.43	0.06638
Oscillospira;s__	2.085 ± 0.119[b]	2.51[a]	-3.03[c]	0.73[b]	-1.79[c]	0.00001
Megasphaera;s__elsdenii	3.386 ± 2.635	-4.05	0.43	-2.23	-0.86	0.54442
Collinsella;s__aerofaciens	2.389 ± 0.747[a]	-2.35[b]	-1.90[b]	-1.44[b]	0.54[a]	0.04331
Eggerthella;s__lenta	0.053 ± 0.015[a]	-1.95[b]	-3.74[b]	-1.63[b]	0.39[a]	0.04445
Coprobacillus;s__cateniformis	0.042 ± 0.027[b]	4.35[a]	4.66[a]	4.00[a]	-2.60[b]	0.00006
Sutterella;s__	1.941 ± 0.595[b]	1.34[a]	0.15[abc]	1.17[a]	-0.17[bc]	0.01943
Prevotella;s__bivia	0.009 ± 0.013	-1.00	-0.72	-0.94	0.01	0.80215
Prevotella;s__disiens	0.047 ± 0.080	-4.16	-3.05	-3.69	-0.08	0.83669

8.3.4. qPCR Prebiotic Index

Considering the results (Figure 1), we found out that the fermented substrate with the best prebiotic activity was FOS after 18 h, and the runner-up was HBPA after 24 h. In comparison to FOS 18 h, HBPA 18 h scored 1.44-fold lower values. The blank control scored for any time points lower values than any HBPA, HB, and FOS cases (all significative, but one) and reached the lowest value of the dataset at the endpoint (26.24-fold lower than FOS 18 h).

So far, the qPI of HBPA leans to reach high level later than the FOS. Anyhow, even at the earlier time points qPI of HBPA was higher than the blank control. Thus, the comparable prebiotic index of HBPA could be mostly due to its high portion of soluble fibers. Similarly to FOS it is known that soluble fibers are excellent substrates for production of SCFAs in the large intestine (Oba et al., 2020).

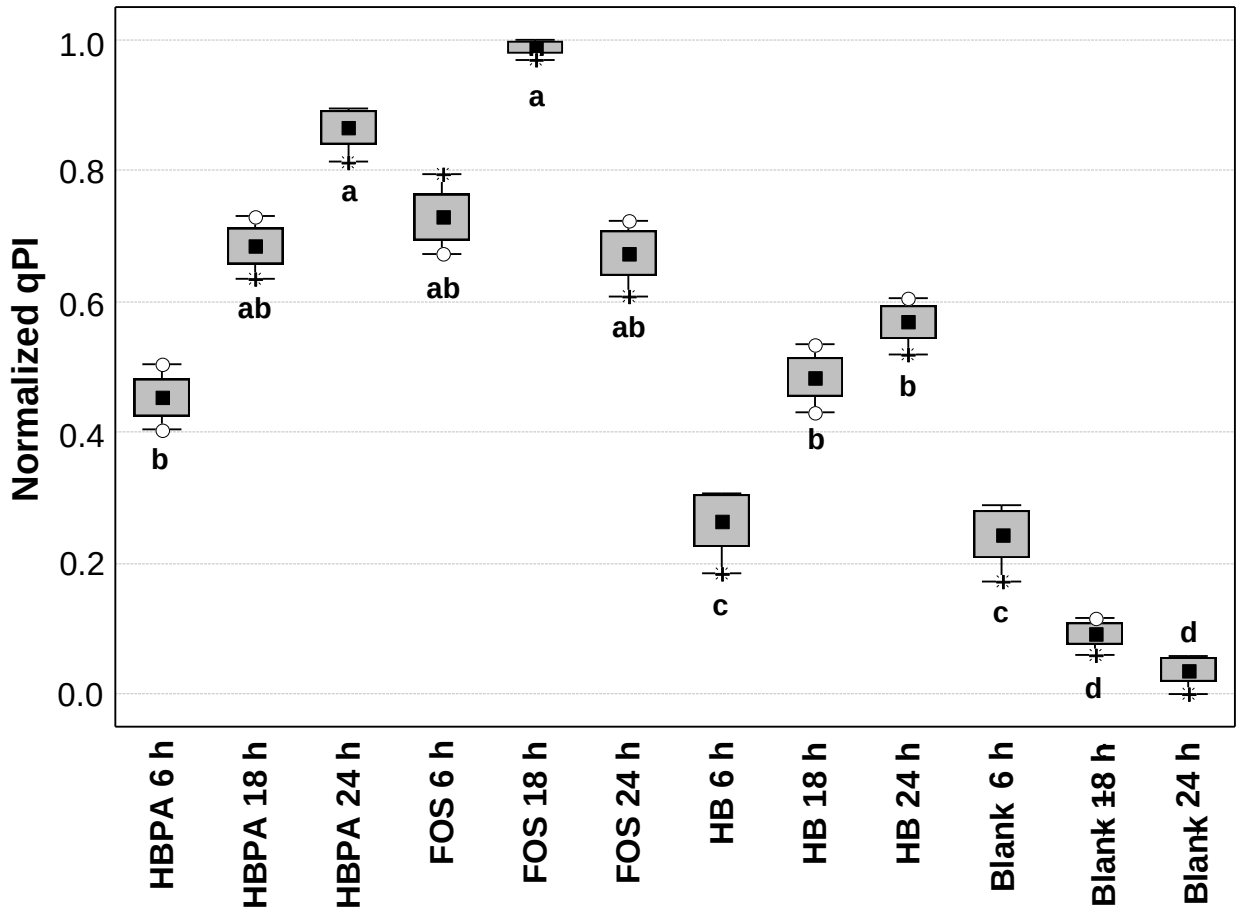

Figure 1. qPCR Prebiotic Index (qPI) of colonic fermentations on the substrates HBPA, HB, FOS, and on a blank control, at different time points. [abcd]Different letters indicate statistical significance by Tukey's honestly significant differences (HSD) test ($p < 0.05$). Marker = mean; box = mean ± S.D.; whiskers = min and max; dots = outliers; asterisks = extremes.

8.3.5. Changes in main microbial metabolites related to prebiotic potential

To analyze the main changes in volatile microbial metabolites related to prebiotic potential, we have considered the shift in loads from the baseline to the endpoint (24 h) of fermentations of 10 selected VOCs (ANOVA $p < 0.05$) with renowned bioactivity in humans (low organic acids and aromatic compounds) as follows: a) each single compound was normalized (mean centering method) within its dataset, which included cases from HB, HBPA, FOS, and the blank control at different time points; b) the baseline dataset (Table S2) was then subtracted to the endpoint dataset; c) *post-hoc* analysis was done to compare the sample productions of a single molecule (Tukey's HSD, $p < 0.05$). The first set of compounds is relative to low organic acids, such as Acetic, Propanoic, Butanoic, Pentanoic, and Hexanoic acids that are beneficial compounds essential for the host, the mucosa, and the colon microbiota itself (Figure 2). The second set is relative to compounds related to proteolytic

fermentation and/or detrimental for the host, such as Indole, Phenol, p-Cresol, Benzaldehyde, and Phenol, 2,4-bis(1,1-dimethylethyl)- (2,4-DTBP) (Figure 3).

From our results (Figure 2), organic acid concentration was increased with HB, HBPA, and FOS, while no changes nor production of any of them was recorded in the blank control. Starting from small amounts detected at the baseline (< 0.010 mg/kg for Acetic, 0.012 mg/kg for Propanoic, 0.101 mg/kg for Butanoic), the capacity to produce Acetic, Propanoic, and Butanoic acids was generally (considering the means of every time points) stronger for FOS than for HB or HBPA ($p < 0.05$). In particular, FOS fermentation accounted for 2.25-, 3.37-, and 4.87-folds more than HBPA, respectively for these three compounds. A reduction in Acetic, Propanoic, and Butanoic acids abundances is linked to dysbiosis of the colon microbiota and a reduced intestinal cell homeostasis (Gibson et al., 2017). The prebiotic activity of HBPA is linked to its capacity to foster *Lactobacillus* spp., *Bifidobacterium* ssp., and *Enterococcus* spp. that metabolize the fibers and produce low organic acids. On the opposite, starting from little amounts at the baseline (< 0.010 mg/kg either for Pentanoic and Hexanoic acids), the surge of Pentanoic and Hexanoic acids was stronger for HBPA than FOS ($p < 0.05$). In details, HBPA fermentation accounted for 1.27- and 2.08-folds more, respectively of these two compounds. Pentanoic and Hexanoic acids are Medium Chain Fatty Acids (MCFAs) are protective on glucose homeostasis and against insulin resistance and are important metabolic biomarkers of dysbiosis and Intestinal Bowel Disease (IBD) (Zhou et al., 2017; Koji et al., 2010; Lundsgaard et al., 2021). The increased abundance in MCFAs observed in this study could be due to the ability of HBPA to foster *Bifidobacteriaceae* and commensals *Clostridium* group IV, or *Bacteroides* spp. Actually, MCFA production by these three bacterial groups happened during fiber fermentation (Riviere et al., 2018).

The ability of HBPA to liberate once fermented more SCFAs than HB could be due to the higher availability of lower MW peptides/proteins and to the higher fermentation preference of these substrates by *Lactobacillales* and *Bifidobacteriaceae*. Similarly, the more of these species were fostered and the more was the ability to elongate MCFAs from lactate production via reverse β-oxidation (Contreras-Davila et al., 2020).

The second set comprised VOCs that had a different trend for the substrates than the blank control (Figure 3). Indole abundances increased with HB, HBPA, and FOS but decreased in BC. Oppositely, Phenol, p-Cresol, Benzaldehyde, and 2,4-(DTBP) were reduced with HBPA and partially with HB, while increased with BC. HBPA was able to produce 2.08-fold more Indole than FOS, and to reduce 1.54- and 1.48-fold more Phenol and 2,4-(DTBP) than FOS, respectively ($p < 0.05$).

Indole is a tryptophan catabolite, deriving from degradation of the proteinaceous portion of the food[5] by commensal *Escherichia coli*. Indole is also suggested to have beneficial effects, such as the attenuation of inflammation indicators on HCT-8 cells at the concentration of 1mM (Bansal et al., 2009). Otherwise, its accumulation as bacterial products (*Clostridium* spp. and *Escherichia* spp.) could result toxic for the host, because if it is not microbially degraded in beneficial derivates (*e.g.* Indole propionic acid) is metabolized into Indoxyl sulphate in the liver that, as the prototype of protein–bound uremic toxins (Taleb et al., 2019), provokes chronic kidney disease and vascular disease (Bansal et al., 2009; Wang et al., 2020). Despite, the dose of indole to generate such detrimental effect is undefined, a study finds that cattle injected with 0.2 g/kg of body weight after 72 h had diarrhoea, haemolysis, haemoglobinuria, and microscopic lesions of haemoglobinuric nephrosis (Hammond et al., 1980).

Similarly, Phenol and p-Cresol are derived from proteolytic fermentation and have been shown to damage epithelial barrier function in vitro and can be potentially carcinogenic (Wang et al., 2020).

From our results (Figure 3), the shifts recorded by FOS and HBPA fermentations compared to the baseline indicated a slight increase in Indole and a reduction in metabolites (phenols) related to animal fat and protein degradation. These compounds were more abundant at the baseline, as derived by fecal samples of omnivores. Their reductions are in line with the results obtained from the microbiota, indicating an increase in those taxa specialized in plant-based fibers fermentation. When comparing the reduction of these detrimental VOCs of HBPA to that of HB, the better action of HBPA could be attributed to higher bioactivity of alcalase treated HB. Indeed, a previous study demonstrated that HBPA low MW proteins or peptides have an antioxidant activity higher than the high MW proteins or peptides of HB (Setti et al., 2020).

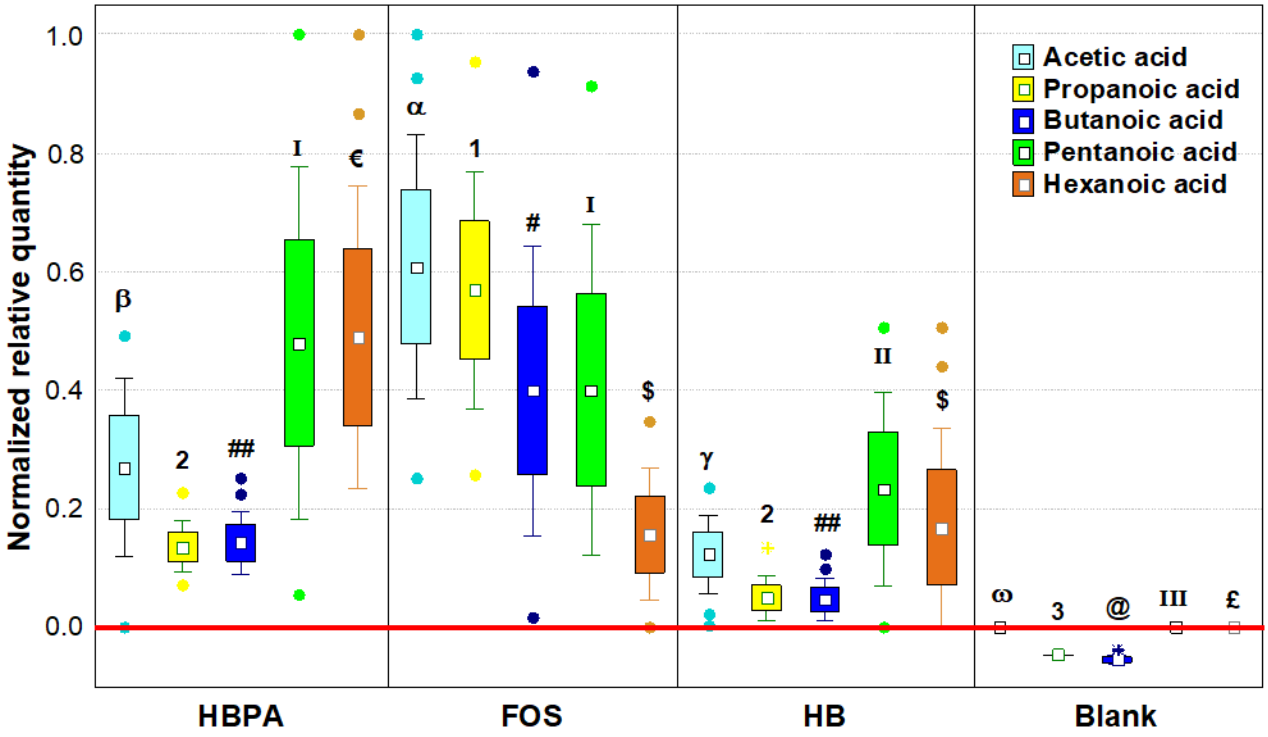

Figure 2. Changes in the abundance of beneficial microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S3). Changes were recorded after 6, 18, and 24 h of *in vitro* fecal batch fermentations with HBPA, HB, FOS, and a blank control. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments (n = 4). Marker = mean; box = mean ± S.D.; whiskers = non outlier range; dots =outliers; asterisks = extremes. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to Tukey's HSD test ($p < 0.05$).

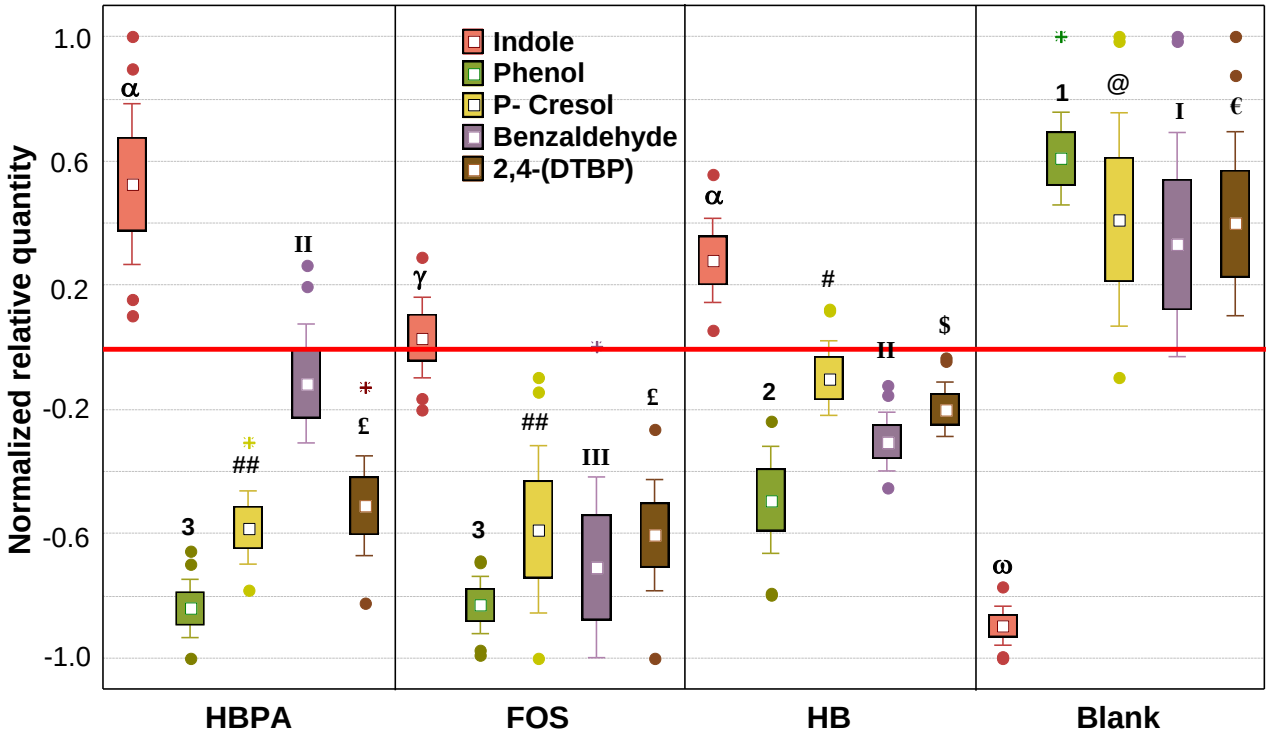

Figure 4. Changes in the abundance of detrimental microbial VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S3). Changes were recorded after 6, 18, and 24 h of *in vitro* fecal batch fermentations with HBPA, HB, FOS, and a blank control. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments ($n = 4$). Marker = mean; box = mean ± S.D.; whiskers = non outlier range; dots =outliers; asterisks = extremes. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to Tukey's HSD test ($p < 0.05$).

8.3.6. Volatilome analysis through SPME GC/MS

Through SPME GC-MS, among 32 duplicated cases (n = 64), 125 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA) (Figure 4). On average, 77 were relatively quantified at the baseline, while 113 were quantified during the 24 h of experiments at different timepoints. For a landscape description of the volatilome, a dataset of 91 significant molecules (ANOVA at $p < 0.05$) was generated and presented as a quantification heatmap (Figure S2), then sorted and super-normalized by chemical classes of VOCs, i.e., organic acids, main detrimental aromatic VOCs, aldehydes, ketones, alcohols, and others (alkenes, alkanes, amines, sulphurates). Organic acids VOCs and detrimental aromatic VOCs were just discussed in paragraph *6.3.6*, as main microbial metabolites related to prebiotic activity, while, from each dataset of the other classes, multivariate analyses, such as untargeted Principal Component Analysis (PCA) and targeted MANOVA ($p < 0.01$) was achieved

to address the specific contributes to VOCs production by the independent variables. Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing one chemical class to another.

A PCA of 27 statistically significant alcohols distributed cases on the plot, separating fermentation with HBPA, HB, FOS, and BC from each other and from the baseline (Figure 4A). From our results, the main descriptors of fermentation with HBPA were mainly complex terpenoid alcohols ($p < 0.01$), such as 4-Terpineol, Beta-Linalool, Cuminol, Eucalyptol, Borneol, and 1,8-Menthadien-4-ol, mainly produced at the intermediate and late time points ($p < 0.01$) while those for FOS were 1-Dodecanol, Propanol, 4-methyl, 3-Buten-1-ol, 3-methyl, and Ethyl alcohol mainly produced at the intermediate time point ($p < 0.01$) (Tables S3–S4). The main descriptor of alcohol production from BC samples remained Isopropyl alcohol ($p > 0.01$). The colon microbiota produces different alcohols during fermentation of dietary polysaccharides. Terpineol, Beta-Linalool, Cuminol, Eucalyptol, and Borneol, that are major terpenoids found in hemp seed with anti-oxidant and anti-inflammatory activity, were increased after lactobacilli fermentation of HB (Nissen et al., 2021).

A PCA of 16 statistically significant aldehydes showed distributed cases on the plot, separating fermentation with HBPA and HB from that made with FOS, and from the blank and the baseline (Figure 2B). The main descriptor of fermentation with FOS was 2-Hexenal ($p < 0.01$), while that for HBPA were Heptanal and 2-Octenal, (E), principally produced at the early time point and at the end point, respectively ($p < 0.01$) (Tables S4 and S5), and lastly the main descriptor of BC was benzeneacetaldheyde, that was present at the baseline, but absent after fermentation with the substrates. Aldehydes are a result of microbial fermentation and lipid oxidation. Certain aldehydes are health-promoters, like 2-Octenal, (E) that was reported to limit the growth of several intestinal pathogens at a very low concentration (Zhang et al., 2020), while most are detrimental, being cytotoxic at a low threshold, such as Benzeneacetaldheyde (Zhang et al., 2020).

A PCA of 16 statistically significant ketones distributed cases on the plot, separating the substrates from each other and from the baseline (Figure 4C). Descriptors of fermentation with HBPA were p-Menthone (77.00%) and Acetophenone (81.00%), majorly produced at the endpoint (77.04% and 51.83%, respectively) ($p < 0.01$) (Tables S3 and S4). The main descriptor of fermentation with FOS was 2,3-Butadione (68,93%), that of HB and BC were 2-Heptanone and Acetone, respectively but not significantly (Table S3). During colonic fermentation, many ketones are produced; considering

their bioactive attributes, some are desirable, such as Acetophenone that acts as antimicrobial to different Gram-negative bacteria, and its N-substitute derivates have been proposed as a therapeutic approach in diabetes (Taslimi et al., 2020). In our experimental condition, Acetophenone is probably derived from the bacterial deconjugation of polyphenols, as *Lactobacillales* (Zhu et al., 2020), which was increased by hydrolytic process in HBPA.

A PCA of 22 statistically significant other VOCs, related to alkenes, alkanes, amines, and sulphurates distributed cases on the plot, separating the substrates from each other and from the baseline (Figure 4D). The main descriptor of fermentation with FOS was Ethyl Acetate ($p < 0.01$), while those for HBPA were Caryophillene and D-Limonene, that for HB was Eicosane, while BL was described by Aniline. These VOCs were discriminated significantly just for the category of substrates ($p < 0.01$) (Tables S4), but no significant differences were detected for the category of time ($p > 0.05$), except for Aniline that was reduced significantly on a time basis ($p < 0.01$) (Table S4). Caryophillene and D-Limonene are potent health-related terpenes (Nissen et al., 2021) and the features observed indicate that the descriptors of HBPA were not subject to fermentation and thus their bioactivity was preserved from the food matrix. Aniline is instead a carcinogen derived from benzenoid pollutants (Zhang et al., 2021), and its reduction by fermentation with HBPA is a positive feature.

CASE STUDY 5: Prebiotic potential of hemp bran and its alcalase hydrolysate

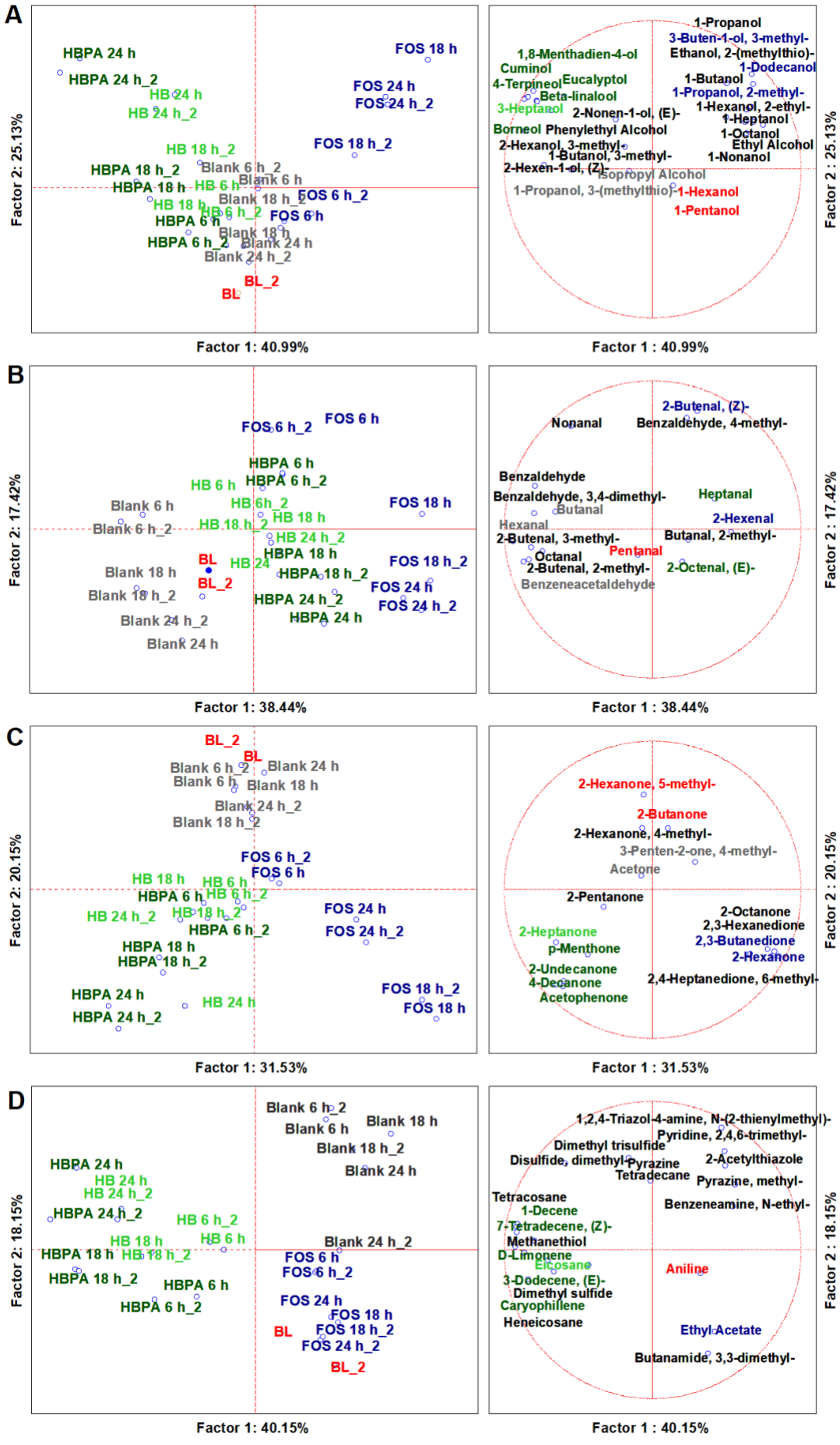

Figure 2. PCAs of the volatilome sorted by chemical classes of significant (ANOVA $p < 0.05$) VOCs, including the biological replicas of HBPA, HB, FOS, the blank control, and the baseline (BL) and three different timepoints (6 h, 18 h, 24 h). **A**) Alcohols; **B**) Aldehydes; **C**) Ketones; **D**) Other VOCs. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of the respective group of cases.

8.3.7. Interomic Correlations among Bioactive Metabolites and the Microbiota

Spearman Rank Correlations ($p < 0.05$), two-joining-way Heatmaps, and Pearson cluster analysis were performed by the comparison of two different normalized datasets, each derived from values of relative quantification (OTUs and VOCs) of the sole HBPA dataset (Figure 5). The significance of correlations is reported in the Figure S3. From the Pearson dendrograms, two main clusters and a smaller one was identified that probably may explain the cause and effect of the prebiotic potential of HBPA. The first cluster related to bacterial taxa included *Bif. bifidum, Bact. fragilis, Bact. thetaiotaomicron, Sutterella spp.,* and *F. prausnitzii,* that have positive correlations with beneficial SCFAs and MCFAs, as well as with bioactives VOCs such as 4-Terpineol, Borneol, Acetophenone and others. This cluster has also negative correlations with detrimental Phenol and p-Cresol. The second cluster included *Colinsella aerofaciens, Blautia obeum, and Bilophila wadsworthia* that have negative correlations with most of the beneficial compounds, and positive correlations with Phenol. These features have been reported by other studies, as this group of bacteria is related to dysbiosis and intestinal syndromes (Liu et al., 2022). Lastly the third small cluster included *Roseburia faecis* and *En. faecalis*, that have positive correlations with most of the beneficial compounds and negative correlations with Phenol. Interestingly, *R. faecis* was the only one positively correlated with Indole, in line with recent findings (Knudsen et al., 2021).

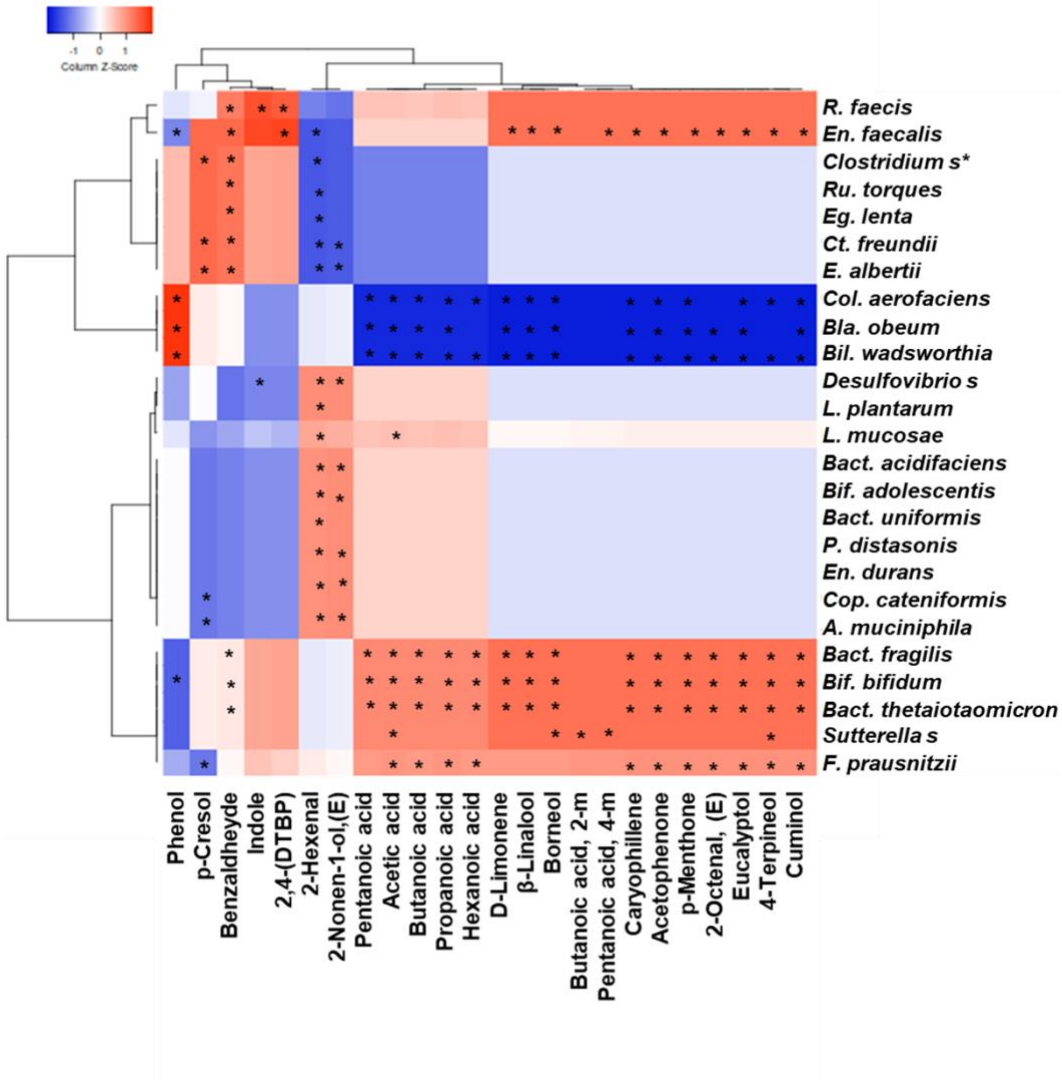

Figure 5. Interomics, Spearman Rank Correlations from the HBPA datasets related to microbial metabolites of the volatilome and species OTUs from the microbiota. Left side dendrogram identifies by Pearson analysis three major different clusters among bacterial species. Significance of correlations are provided as supplementary material (Figure S4).

8.4. Conclusions

Based on the positive results obtained by different beneficial (F/B ratio, microbial diversity, organic acids) or harmful (Phenol, P-Cresol, etc.) indicators, our study evidenced that HB and in particular HBPA had a prebiotic potential comparable to that of FOS. Furthermore, the populations of beneficial and fiber degrading bacteria were fostered and in contrast those of opportunistic and proteolytic bacteria were limited by HBPA and minorly by HB colonic fermentations. Additionally, alcalase treatment of HB makes a product more potent, in terms of prebiotic activity probably due to a higher release of small peptides that along with being more bioactive directly on the host (i.e. antioxidant and antihypertensive) are also more accessible and specific as substrates for the fermentation by beneficial microbes, and nasty or even toxic for the fermentation by opportunistic microbes.

CASE STUDY 5: Prebiotic potential of hemp bran and its alcalase hydrolysate

The use of MICODE, a robust and versatile *in vitro* model together with multivariate statistics visibly demonstrated a suitable approach to describe the effects generated by the alcalase hydrolysis and to explain the prebiotic potential of hydrolysates.

Such *in vitro* approach could be included in a pipeline of experiments where a reduced number of animals for testing is employed, according to the Directive 2010/63/EU and the Regulation (EU) 2019/1010. To fully understand the efficacy of HBPA on human health a diet intervention study is imperative, and the results presented are target-effective and should have robustness for pre-clinical applications.

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8.6. Supplementary materials

Table S1. Primers pairs employed for PCR and qPCR reactions and quantifications.

Group	Target	Sequence 3'-5'	Bp*	Reference
Eubacteria	V3-V4 16 S	Eub518-R: ATTACCGCGGCTGCTGG Eub338-R: ACTCCTACGGGAGGCAG	147	Lane et al., 1992
Enterobacteriaceae	V3-V4 16 S	Enterobac-F: TGCCGTAACTTCGGGAG Enterobac-R: TCAAGGACCAGTGTTCAG	450	Bartosch et al., 2004
Lactobacillales	V3-V4 16 S	Lac-F: GCAGCAGTAGGGAATCT Lac-R: GCATTYCACCGCTACACA	340	Walter et al., 2001
Bifidobacteriaceae	RecA	RecA-F: CGTYTCBCAGCCGGAYA RecA-R: CCARVGCRCCGGTCATC	220	Masco et al., 2004

Clostridium group I	V3-V4 16 S	ClosI-F: TACCHRAGGAGGAAGCCAC ClosI-R: GTTCTTCCTAATCTCTACGCAT	148	Bartosch et al., 2004

*Base pairs

Table S2. Quantification of VOCs by SPME GC/MS related to prebiotic potential, employing 10000 mg/kg of 2-Pentanol, 4-methyl.

VOCs	**mg/kg ± S.D.**	***p* value**[†]
Acetic acid	traces*	0.0010
Propanoic acid	0.012 ± 0.015	0.0467
Butanoic acid	0.101 ± 0.086	0.0424
Pentanoic acid	traces	0.0153
Hexanoic acid	traces	0.0179
Indole	7.955 ± 1.388	<0.0001
Phenol	0.177 ± 0.051	<0.0001
Phenol, 4-methyl-	15.022 ± 9.808	0.0141
Benzaldehyde	0.717 ± 0.415	0.0013
2,4-(DTBP)**	1.826 ± 0.624	0.0074

*traces < 0.01 mg/kg; **Phenol, 2,4-bis(1,1-dimethylethyl)-; (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg); [†] *p* value of ANOVA from a dataset including all cases and time points.

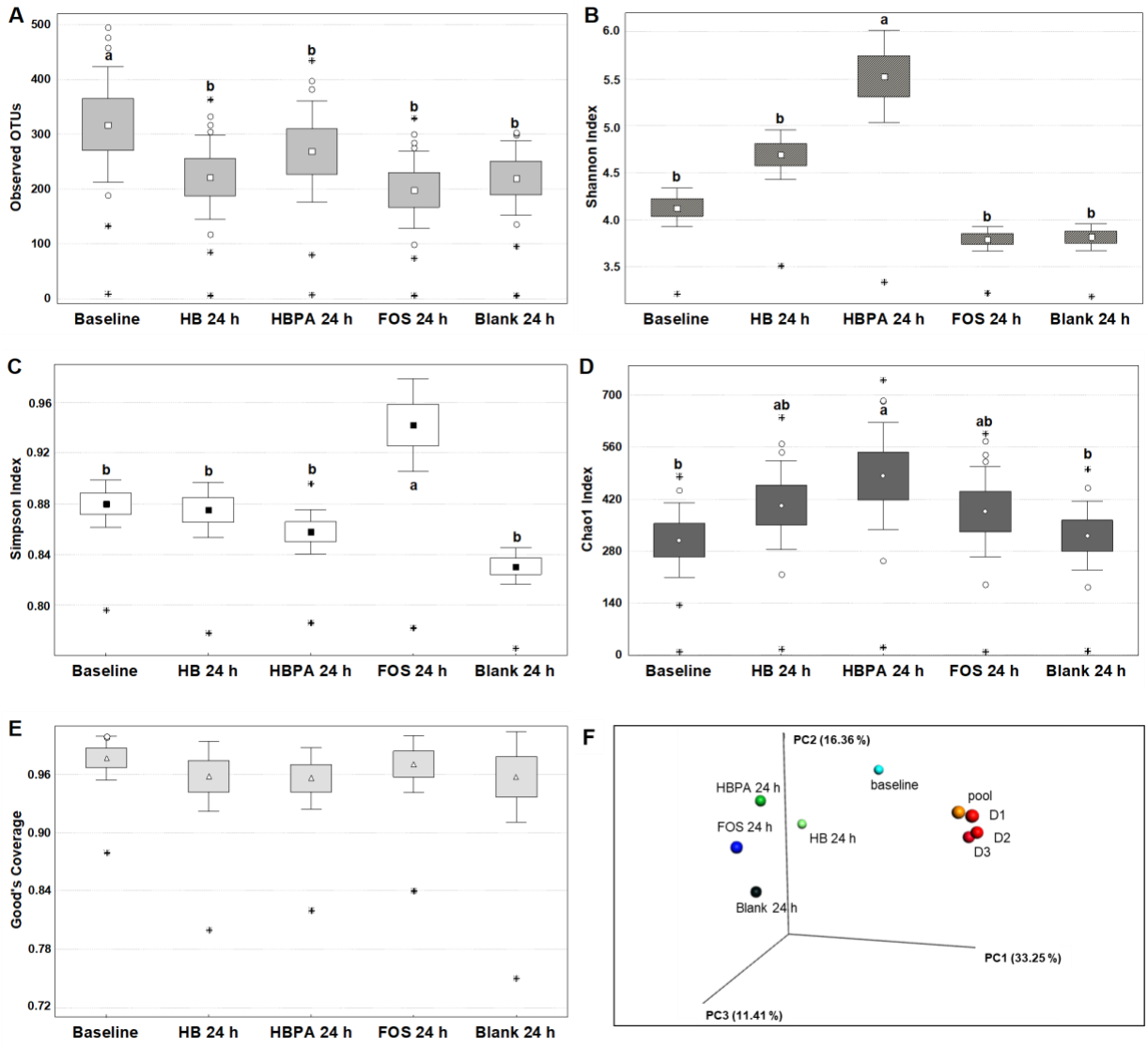


Figure S1. Microbiota alpha and beta diversities. A – E = alpha diversity indexes: F = beta diversity Bray Curtis PCoA. Box = mean ± Standard Error; whiskers = Confidence Interval (95%); dots = Outliers; asterisks = extremes. [ab] Different letters on the plots indicates statistical significance by Student's t-test ($p < 0.05$).

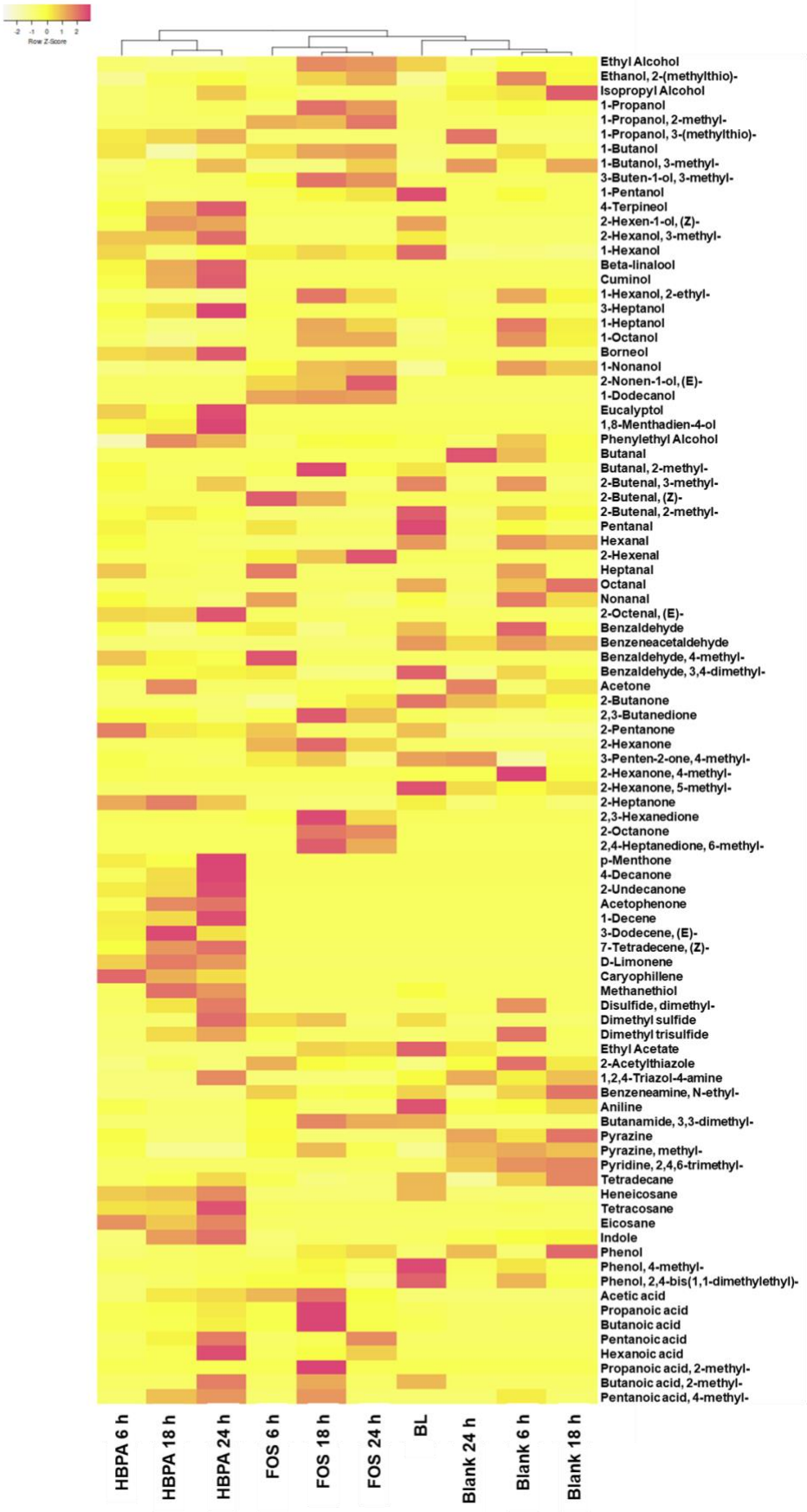


Figure S2. Quantification heatmap of total VOCs.

Table S3. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors significant among the food matrices.

VOC	Baseline	HBPA	FOS	HB	Blank	*p* value*
Alcohols						
Isopropyl Alcohol	0.00^{d}	23.89^{b}	9.06^{c}	7.03^{c}	60.02^{a}	0.07342
1-Propanol	9.90	10.42	48.40	10.23	21.04	0.02754
1-Propanol, 2-methyl-	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.00551
3-Buten-1-ol, 3-methyl-	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.00024
1-Pentanol	59.14^{a}	4.32^{c}	15.31^{b}	11.23^{b}	10.00^{b}	<0.00001
4-Terpineol	0.00^{c}	72.11^{a}	0.00^{c}	27.81^{b}	0.00^{c}	0.00037
2-Hexen-1-ol, (Z)-	57.79^{a}	30.11^{b}	0.00^{d}	12.10^{c}	0.00^{d}	0.00005
2-Hexanol, 3-methyl-	32.00^{ab}	48.00^{a}	0.00^{c}	20.00^{b}	0.00^{c}	0.00002
1-Hexanol	55.73^{a}	14.29^{b}	20.01^{b}	7.46^{c}	2.51^{d}	0.00003
Beta-linalool	0.00^{c}	64.52^{a}	0.00^{c}	35.48^{b}	0.00^{c}	0.00018
Cuminol	0.00^{c}	72.55^{a}	0.00^{c}	27.45^{b}	0.00^{c}	0.00155
3-Heptanol	12.95^{c}	53.83^{a}	3.11^{d}	30.11^{b}	0.00^{e}	0.02945
Borneol	0.00^{c}	69.00^{a}	0.00^{c}	31.00^{b}	0.00^{c}	0.01078
1-Nonanol	0.00^{c}	10.15^{b}	30.91^{a}	14.02^{b}	44.92^{a}	0.00427
2-Nonen-1-ol, (E)-	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.00037
1-Dodecanol	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	<0.00001
Eucalyptol	0.00^{c}	52.88^{a}	0.00^{c}	47.12^{b}	0.00^{c}	0.00569
1,8-Menthadien-4-ol	0.00^{c}	62.47^{a}	0.00^{c}	37.53^{b}	0.00^{c}	0.01391
Aldehydes						
Butanal	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	100.00^{a}	0.00864
2-Butenal, 3-methyl-	62.05^{a}	11.18^{b}	0.00^{c}	8.13^{b}	18.65^{b}	0.00698
2-Butenal, (Z)-	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.00968
2-Butenal, 2-methyl-	68.14	10.10^{bc}	0.00^{c}	5.17^{b}	16.59^{b}	<0.00001
Pentanal	80.23^{a}	5.86^{b}	7.50^{b}	2.13^{b}	4.28^{b}	0.00004
Hexanal	56.00^{a}	8.21^{bc}	0.55^{c}	1.10^{b}	34.14^{a}	0.00098
2-Hexenal	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.00036
Octanal	56.44^{a}	0.00^{b}	0.00^{b}	0.00^{b}	43.56^{a}	0.00412
2-Octenal, (E)-	0.00^{c}	61.12^{a}	0.00^{c}	38.88^{b}	0.00^{c}	0.00012
Benzeneacetaldehyde	56.92^{a}	0.00^{b}	0.00^{b}	0.00^{b}	43.08^{a}	<0.00001
Benzaldehyde, 3,4-dimethyl-	60.93^{a}	12.44^{b}	7.49^{bc}	5.14^{c}	14.00^{b}	0.00049
Ketones						
2,3-Butanedione	10.81^{bc}	11.12^{abc}	68.93^{a}	6.12^{b}	3.02^{c}	0.03688
2-Pentanone	42.65^{a}	24.40^{b}	18.54^{b}	14.40^{b}	0.00^{c}	0.00218
2-Hexanone	0.86^{c}	4.08^{b}	94.70^{a}	0.00^{c}	0.35^{c}	<0.00001
2-Hexanone, 5-methyl-	78.16^{a}	0.00^{c}	0.00^{c}	0.00^{c}	21.84^{b}	<0.00001
2-Heptanone	29.54^{b}	40.13^{a}	1.11^{c}	25.00^{b}	4.23^{c}	0.00001
2-Octanone	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.00549
2,4-Heptanedione, 6-methyl-	0.00^{b}	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.01297
p-Menthone	0.00^{c}	77.00^{a}	0.00^{c}	23.00^{b}	0.00^{c}	0.04972

2-Undecanone	0.00^{c}	67.84^{a}	0.00^{c}	32.16^{b}	0.00^{c}	0.02230
Acetophenone	0.00^{c}	81.00^{a}	0.00^{c}	29.00^{b}	0.00^{c}	0.00115
Others (alkanes, alkenes, sulfurates, amines, …)						
1-Decene	0.00^{c}	60.40^{a}	0.00^{c}	39.60^{b}	0.00^{c}	0.00185
3-Dodecene, (E)-	0.00^{c}	70.96^{a}	0.00^{c}	29.04^{b}	0.00^{c}	0.00391
7-Tetradecene, (Z)-	0.00^{c}	80.00^{a}	0.00^{c}	20.00^{b}	0.00^{c}	0.00089
D-Limonene	0.00^{c}	69.77^{a}	0.00^{c}	20.23^{b}	0.00^{c}	<0.00001
Caryophillene	0.00^{c}	82.10^{a}	0.00^{c}	17.90^{b}	0.00^{c}	0.00008
Methanethiol	22.80^{b}	56.20^{a}	0.00^{c}	21.00^{b}	0.00^{c}	0.02011
Ethyl Acetate	68.04^{a}	0.00^{c}	20.36^{b}	0.00^{c}	11.61^{bc}	0.00001
Aniline	70.63^{a}	3.02^{c}	4.80^{c}	3.09^{c}	18.46^{b}	0.00143
2-Acetylthiazole	0.00^{c}	6.61^{b}	29.60^{a}	10.01^{b}	53.77^{a}	0.00907
Butanamide, 3,3-dimethyl-	52.99^{a}	0.00^{b}	40.11^{a}	6.90^{b}	0.00^{b}	0.00029
Pyrazine	0.00^{c}	9.23^{b}	10.50^{b}	7.25^{b}	73.01^{a}	0.00020
Pyrazine, methyl-	0.00^{d}	8.10^{c}	25.30^{b}	16.00^{bc}	50.60^{a}	0.02990
Pyridine, 2,4,6-trimethyl-	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	100.00^{a}	<0.00001
Heneicosane	28.73^{a}	51.16^{a}	0.00^{c}	20.11^{b}	0.00^{c}	<0.00001
Tetracosane	0.00^{c}	77.17^{a}	0.78^{c}	20.30^{b}	1.75^{c}	0.00050
Eicosane	0.00^{b}	55.00^{a}	0.00^{b}	45.00^{a}	0.00^{b}	0.00002

*p value indicates MANOVA significance. ^{abcd} Different letters indicate statistical significance by Tukey's HSD test ($p < 0.05$).

Table S4. MANOVA categorical descriptors for the volatilome, categorized for the time of fermentation. % of contribution of VOCs descriptors significant among the time points.

VOC	**0 h %**	**6 h %**	**18 h %**	**24 h %**	***p* value***
Alcohols					
1-Propanol, 3-(methylthio)-	0.00^{c}	14.19^{b}	18.65^{b}	67.16^{a}	0.04120
1-Butanol, 3-methyl-	0.00^{cd}	6.64^{c}	28.59^{b}	64.77^{a}	0.00222
1-Pentanol	70.37^{a}	9.42^{b}	8.90^{b}	11.30^{b}	<0.00001
2-Hexen-1-ol, (Z)-	57.79^{a}	2.97^{c}	20.67^{b}	18.57^{b}	0.09274
1-Hexanol	55.73^{a}	18.48^{b}	12.24^{b}	13.54^{b}	0.00628
1-Nonanol	0.00^{b}	32.78^{a}	36.83^{a}	30.39^{a}	0.03760
1-Dodecanol	0.00^{c}	11.79^{bc}	54.73^{a}	33.48^{ab}	0.04820
Eucalyptol	0.00^{c}	8.24^{bc}	25.72^{b}	66.04 ^{a}	0.04132
1,8-Menthadien-4-ol	0.00^{c}	11.78^{b}	14.07^{b}	74.15^{a}	0.04173
1-Propanol, 3-(methylthio)-	0.00^{c}	14.19^{b}	18.65^{b}	67.16^{a}	0.04120
1-Butanol, 3-methyl-	0.00^{c}	6.64^{c}	28.59^{b}	64.77^{a}	0.00222
Aldehydes					
2-Butenal, 3-methyl-	62.05^{a}	24.15^{b}	1.68^{c}	12.13^{c}	0.00626
2-Butenal, 2-methyl-	68.14^{a}	15.05^{b}	13.11^{bc}	3.70^{c}	0.03809
Pentanal	82.36^{a}	17.64^{b}	0.00^{c}	0.00^{c}	<0.00001
Hexanal	56.00^{a}	25.44^{b}	17.68^{b}	0.87^{c}	0.01621
Heptanal	0.00^{b}	92.92^{a}	5.97^{b}	1.11^{b}	0.00008

Nonanal	22.39[ab]	53.78[a]	19.35[b]	4.47[b]	0.00254
Benzaldehyde	40.29[a]	38.00[a]	7.75[b]	13.97[ab]	0.01244
Ketones					
3-Penten-2-one, 4-methyl-	41.56[a]	14.62[b]	21.60[ab]	22.22[ab]	0.02732
2-Hexanone	0.00[b]	14.70[b]	70.08[a]	15.22[b]	0.02009
2-Hexanone, 5-methyl-	78.16[a]	5.23[b]	8.05[b]	8.56[b]	0.00013
4-Decanone	0.00[b]	0.00[b]	21.75[ab]	78.25[a]	0.03151
2,4-Heptanedione, 6-methyl-	0.00[b]	0.00[b]	62.75[a]	37.25[ab]	0.04127
p-Menthone	0.00[b]	16.15[b]	6.81[b]	77.04[a]	0.04913
Acetophenone	0.00[b]	2.96[b]	45.21[a]	51.83[a]	0.04298
Others					
Ethyl Acetate	68.04[a]	3.10[b]	11.93[b]	16.93[b]	0.00010
2-Acetylthiazole	0.00[b]	52.54[a]	28.34[ab]	19.12[ab]	0.10464
Aniline	73.63[a]	13.05[b]	9.43[b]	3.89[b]	0.00321
Eicosane	0.00[b]	26.72[ab]	23.97[ab]	49.31[a]	0.04948

*p value indicates MANOVA significance. [abcd] Different letters indicate statistical significance by Tukey's HSD test ($p < 0.05$).

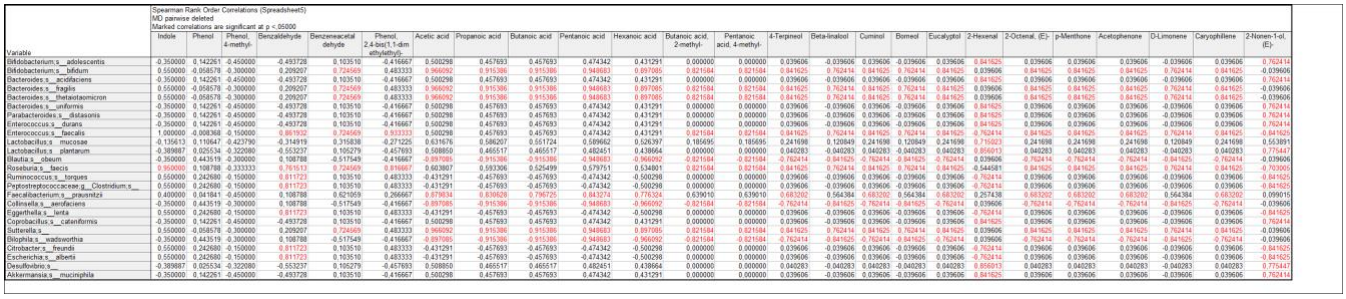


Figure S3. Significance of Spearman rank correlations.

9. CASE STUDY 6: Colonic *in vitro* model assessment of the effect of lactose-free milk on gut microbiota of lactose intolerant and healthy donors

(Lactose intolerants published in International Journal of Food Science and Technology 16/12/2022. DOI: https://doi.org/10.1111/ijfs.16253)
(Healthy subjects submitted to Microorganisms 31/12/2022)

9.1. Introduction

A staggering 4000 million people cannot digest lactose properly (Campbell et al., 2005). Although the strategy adopted to treat lactose intolerance mainly relies on the use of lactose-free products, the possibility of a probiotic-based treatment is gaining momentum given the central role of human colon microbiota (HCM). because it is central the role of lactose-degrading microbial enzymes.

At present, the few clinical studies investigating the effect of probiotics administration in lactose-intolerant adults showed alleviation of symptoms (Cano-Contreras et al, 2022; Vitellio et al., 2019; Pakdaman et al, 2015; He et al, 2008), but the number of different probiotics species and their combination make a systematic study in humans almost impossible. Although clinical studies remain the gold standard, a valid tool would be needed to pre-screen the most effective species/combinations to be then tested in humans. Animals (Xue et al., 2020; Alexandre et al., 2013) are not a valid tool since they have a different microbiota, and this makes difficult to translate results to humans. Even in humanized animals (Ntemiri et al. 2019), the model is based on microbiota from elderly, which does not reflect that of an adult (Kim & Jazwinski 2018). In addition, science is moving towards a drastic reduction in animal experimentation.

In this light, the development of suitable in vitro models is increasingly necessary. In the literature, the few studies on lactose intolerance carried out using in vitro models used HCM from healthy donors (Makivuokko et al., 2006; Windey et al., 2015), whose gut microbiota may be deeply different from lactose intolerants. Moreover, most of the aforementioned studies used pure compounds (lactose) and not food matrices (milk or dairy products).

In the present study, to assess the impact of milk with and without lactose we propose an in vitro model for gastric digestion and colonic fermentation based on the HCM of lactose-intolerant adults and of healthy adults. We combined the INFOGEST digestion protocol to gut fermentation in

MICODE model (Multi Unit In vitro Colon Model) and studied the perturbations in microbiota composition and their metabolites to obtain data with preclinical robustness.
Considering this all, in our microbiology-oriented study we propose an *in vitro* model for gastric digestion and colonic fermentation based on the HCM of lactose-intolerant adults and of healthy adults to assess the impact of milks with and without lactose-containing and lactose-free dairy products. We coupled an INFOGEST digestion to MICODE (Multi Unit In vitro Colon Model) fermentation model and studied variations in colonic microbiota composition and production of key microbial metabolites to obtain data with preclinical robustness.

9.2. Materials and methods

9.2.1. *Human Colon Microbiota*

HCM was obtained from the stools of two lactose-intolerant volunteers and two healthy volunteers. The volunteers were adults not consuming antibiotics, pre- or probiotic supplements in the 3 months prior to the experiment, normal weight, non-smokers, and with no history of chronic gastrointestinal disorders. The lactose-intolerant volunteers were adults with positivity to lactose breath test. Volunteers were informed of the purposes and procedures of the study and provided their written informed consent, in accordance with the Ethics Committee of the University of Bologna.
Human stools were collected by volunteers in a dedicated sterile container, placed in an anaerobic jar with oxygen catalyst (Oxoid, Thermo Fisher Scientific, USA), transferred to the laboratory, and processed within 2 h. HCM was obtained by homogenizing 2 g of each donation in 36 mL of pre-reduced phosphate buffered saline (PBS) (Wang et al, 2020; Nissen et al, 2022).

9.2.3. In vitro digestion and fermentation

UHT semi-skimmed milk (L) and UHT semi-skimmed lactose-free milk (LF) were purchased from Granarolo S.p.A. (Bologna, Italy). Milks were digested in vitro following the INFOGEST protocol (Minekus et al., 2014) and the digestates were then stored at -80 °C. Prior to in vitro colonic fermentation, the digestates were thawed and gently centrifuged to precipitate the denser portion.
Short-term batch proximal colon fermentations were conducted for 24 hours in independent vessels using an in vitro colon model, MICODE (Nissen et al., 2021; 2021a; 2022; 2022a). The preparation of the experiments was made according to published procedures (Connolly et al., 2012; Koutsos et al., 2017; Wang et al., 2020) and described in detail in Nissen et al. (2021; 2021a; 2022). Briefly, fermentation vessels were filled aseptically with 90 mL of basal medium (Connoly et al., 2012; Koutsos et al., 2017; Diotallevi et al., 2021) and left running to reach and maintain the proximal colon

ecological conditions (0.0% of DO2 and pH 6.75). Afterwards, the three different bioreactors were loaded with 9 mL of fecal slurry (10% w/v of human feces in O2 reduced PBS) and: i) 1 mL of LF; ii) 1 mL of L; or iii) 1 mL of deactivated digestive enzymes as the blank control (BC). After adaptation to the ecological conditions, considered as the baseline (BL) corresponding to 1.52 ± 0.18 h, the batch cultures were run under controlled conditions and sampled at different time points (BL; intermediate point (T1) = 18 h; end point (EP) = 24 h) (Nissen et al. 2021a; 2022).

9.2.4. DNA extraction, amplification, and sequencing

DNA was extracted from the fecal samples and from the MICODE effluents at each time points using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

Enumeration of bacterial groups was made with DNA by qPCR to evidence changes in the microbiota after fermentation following previous protocols (Modesto et al., 2011; Tanner et al., 2014; Tamargo et al., 2022; Nissen et al., 2021a; 2022; 2022a). The changes in the abundances of 6 bacterial targets (*Eubacteria*, *Firmicutes*, *Bacteroidetes*, *Lactobacillales*, *Bifidobacteriaceae*, and *Enterobacteriaceae*) (Table S1) were assessed by qPCR on QuantStudio 5 System (Applied Biosystem, Thermo Fisher, Waltham, MA, USA). The shifts in abundance of qPCR values in respect to the BL were calculated as Log2(F/C) (Love et al., 2014). Technical replicas of analyses were conducted in triplicate.

Metataxonomy was conducted through16S-rDNA sequencing by IGA Technology Service Srl (Udine, Italy). Libraries were sequenced with MiSeq (Illumina Inc, San Diego, CA., USA) in paired end with 300-bp read length (Marino et al., 2019). Sequence data analysis was conducted according to previously published papers (Marino et al. 2019; Nissen et al., 2021). Technical replicas of analyses were conducted in duplicate for the BL and in pooled samples for the endpoints.

9.2.5. Volatilome analysis

Volatile organic compounds (VOCs) evaluation was performed on a Gas Chromatograph (7890A, Agilent, Santa Clara, CA, USA) Mass Spectrometer (5975, Agilent, USA) operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME (Solid Phase Micro-Extraction) GC-MS protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Nissen et al., 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Preconditioning, absorption, desorption of SPME–GC-

MS analysis, and all data processing were carried out according to literature (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021). Molecules Identification was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). The VOCs were then relatively quantified (Peak Area %), sorted for respective chemical class, i.e., organic acids, alcohols, and other VOCs, and normalized (Nissen et al., 2021b). In samples at BL the main microbial VOCs related to fermentation of foods were absolutely quantified in mM by SPME GC-MS with the use of an internal standard (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021b; Di Cagno et al., 2011; Casciano et al., 2021). Changes of main microbial VOCs at T1 and EP of fermentations were evaluated in respect of the BL values. Technical replicas of analyses were conducted in duplicate.

9.2.6. Statistical analysis

Normality by Shapiro Wilk's test and Homoscedasticity of Variance by Levene's test were used for the datasets of the volatilome and the qPCR values. Multivariate ANOVA (MANOVA) model by time and matrix categories, followed by post hoc Tukey HSD test ($p < 0.05$), was used to statistically analyze the datasets of volatilome and qPCR values. Principal Component Analysis (PCA) was also computed for the datasets of the volatilome. Statistic of metataxonomy was assessed following the QIIME pipeline version 2.0 (Bolyen et al., 2019). ANOVA model for time category was used for filtered OTUs (open taxonomic units). Statistic was performed with Statistica v.8.0 (Tibco, Palo Alto, CA, USA).

9.3. Results and discussion

9.3.1. LACTOSE INTOLERANTS

9.3.1.1. *Volatilome analysis*

Through SPME GC-MS, 57 molecules were identified with more than 80% of similarity with NIST 11 MSMS library (NIST, USA) and presented as a quantification heatmap (Figure S1). The volatilome was subject to MANOVA model and variables selected, sorted by chemical class, and computed for PCAs.

A PCA of 11 organic acids distributed cases on the plot, separating the BL from T1 and EP of any substrates (Figure 1A). From our results, the main descriptor of fermentation with LF was Butanoic acid (MANOVA 67.17%) (Table S2). The main descriptors of L were Pentanoic, Hexanoic, and Octanoic acids (71.78%, 65.63%, 52.40%, respectively) (Table S2), with Pentanoic and Octanoic acids mainly produced at EP (58.59% and 66.15%, respectively) (Table S3). It is known that

Hexanoic acid is formed by lactose fermentation and free fatty acids lipolysis (Wang et al., 2022), in fact in LF its production was absent (Table S2).

A PCA of 11 alcohols distributed cases on the plot, separating the BL from time points of colonic fermentations and discriminating L at the EP from the others (Figure 1B).

The descriptors of L were 1-Butanol (63.36%) and Phenol (71.57%), while those of LF were Ethyl alcohol (51.07%), 1-Octanol (42.04%), and 1-Hexanol, 2-ethyl- (51.60%) (Table S2).

These molecules were mainly produced at the EP of fermentation (Table S3). It is interesting to note that, according to Windey et al. (2015), a higher number of alcohols are produced during fermentation of lactose. In fact, in our study there was a higher speciation of alcohols with fermentation of L in respect to that of LF.

A PCA of 11 other VOCs distributed the cases over the plot, with poor discrimination on the basis of samples with the exception of L at the EP and BC at T1 (Figure 1C). VOCs that defined L was 2-Hexanone (35.71%) (Table S2), while LF was described by 2-Acetylthiazole (46.54%) (Table S2), exclusively derived from the fermentation process (Table S3).

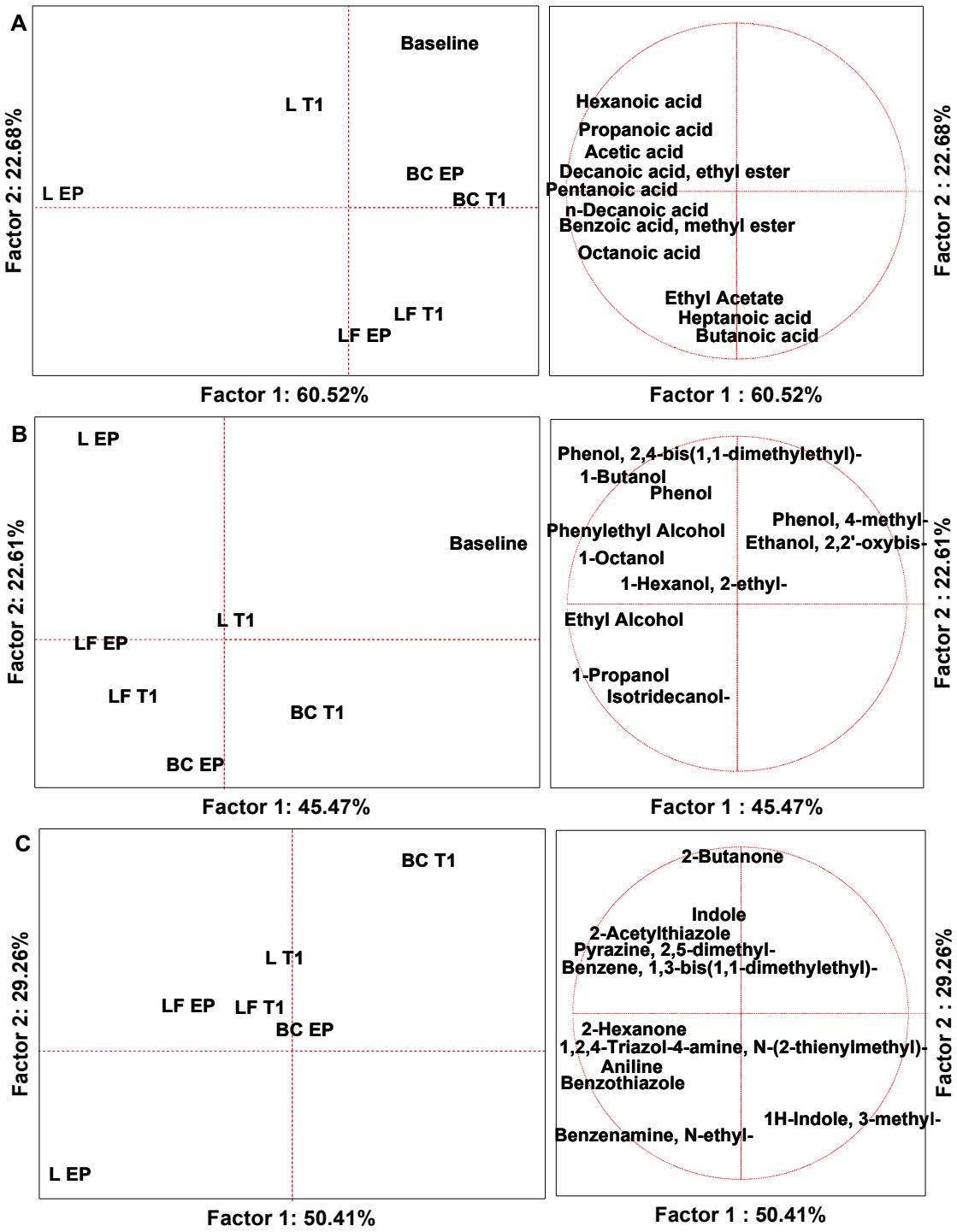


Figure 1. PCA plots of the volatilome sorted by chemical classes, including the biological replicas of L, LF, BC, and the baseline (BL) and different time points (T1 = 18 h and EP = 24 h). A) Acids; B) Alcohols; C) Other VOCs. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. L = milk with Lactose; LF = milk Lactose-Free; BC = Blank Control.

9.3.1.2. Changes in abundance of main microbial VOCs

The baseline values of quantification of acetic acid, propanoic acid, and butanoic acid in our samples (Table S4) were in the mM range of that recorded with similar approaches in feces of lactose intolerant adults (Windey et al., 2015). From our results with respect to BL (Figure 2A), the concentration of acetic acid increased significantly just after L fermentation that in comparison with LF was 2.18 times higher ($p = 0.008120$; Table S2). It is known that when lactose reaches the colon, it acts as a prebiotic and increases the level of carbohydrate fermentation. In fact, the colonic metabolism of lactose has been reported to be associated with an increased production of short-chain fatty acids (Alexandre et al., 2013). In our dataset butanoic acid is depleted with L fermentation, although not significantly in comparison with LF. Because butanoic acid is mainly produced by a healthy colon microbiota, it is clear that the lactose insult has affected those taxa butyrate-producers.

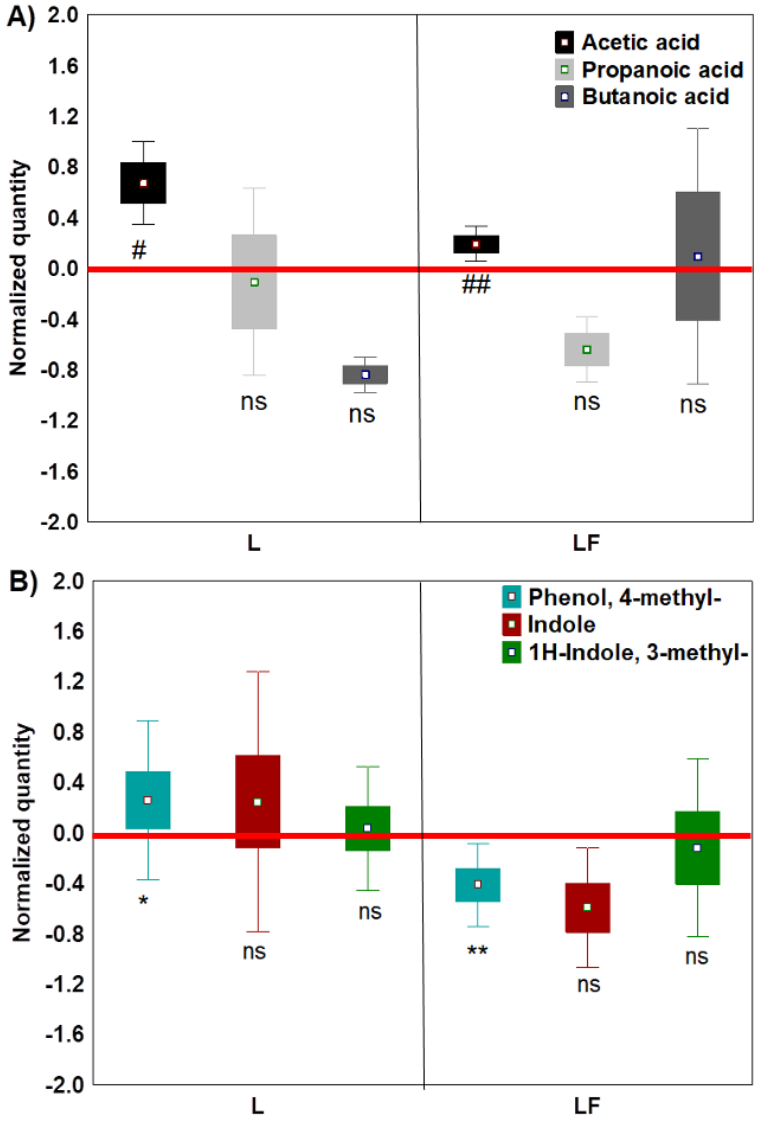


Figure 2. Changes in the abundance of A) beneficial and B) detrimental microbial VOCs in respect to the baseline of in vitro fermentation (red line). Box plots are including all replicas of T1 and EP

values. Marker = mean; box = mean ± standard error; whiskers = mean ± standard deviation. Different symbols among a single independent variable indicate significant difference according to MANOVA model followed by post hoc Tukey's HSD test. ns = not significant; L = milk with lactose; LF = milk lactose free.

Milk is rich in aromatic amino acids, that are largely metabolized by *Proteobacteria* producing detrimental compounds for human health, as Phenol, Indole, Phenol, 4-methyl- (aka p-cresol), and 1H-Indole, 3-methyl (aka skatole) (Wang et al., 2020). Generally, p-cresol concentrations measured in human faeces are rather variable (Wang et al., 2020), but our values (Table S5) were comparable with those observed by Windey et al. (2015).

From our results, fermentation of L increased and LF decreased the production of harmful VOCs, (Figure 2B). In particular, fermentation with L produced about 1.5 times more p-cresol when compared with L ($p = 0.000855$) (Table S2). This result agreed with the characterization of HCM reported below, where *Proteobacteria* increased more after L than with LF fermentations.

9.3.1.3. Changes in abundance of selected bacterial targets absolutely quantified by qPCR

Considering *Eubacteria, Firmicutes*, and *Bacteroidetes* (Table 1), fermentations of both kinds of milk decreased their abundances, with LF as the strongest. Although at EP, LF was significantly stronger than L just for *Bacteroidetes*.

Among the beneficial bacteria, at the EP both milks reduced *Bifidobacteriaceae*, while L increased and LF decreased *Lactobacillales*. Among them, lactic acid bacteria are known to be involved in lactose intolerance relief (Pakdaman et al., 2015), due to their β-galactosidase activity. In accordance, this taxon grew more with L fermentation for the presence of lactose. These results were also seen in an old *in vivo* study by Ito & Kimura (1993), where the authors showed an increase in lactobacilli and bifidobacteria after brief exposure to lactose in lactose intolerant adults.

From our results both milk samples fostered the growth of opportunistic *Enterobacteriaceae* on a time dependency.

Table 1. Enumeration of selected bacterial targets by qPCR.

qPCR Target	**Quantifications**	**Changes**		**MANOVA**
	Cells/mL ± SD	**Log_2(F/C)**		
Eubacteria	**BL**	**T1**	**EP**	
L	2.07E+09 ± 7.68E+07	0.08^{A}	-0.28	0.128684
LF	2.07E+09 ± 7.68E+07^{a}	-0.86^{bC}	-0.31^{a}	0.001363
BC	2.07E+09 ± 7.68E+07	-0.30^{B}	-0.43	0.062125
		0.000133	0.698152	*p* value

Firmicutes	**BL**	**T1**	**EP**	
L	1.60E+09 ± 8.40E+07^{b}	-1.65^{aB}	-3.10^{aB}	0.007940
LF	1.60E+09 ± 8.40E+07^{b}	-3.83^{aB}	-3.70^{aB}	0.000392
BC	1.60E+09 ± 8.40E+07	0.40^{A}	0.02^{A}	0.633917
		0.000011	0.000347	*p* value
Bacteroidetes	**BL**	**T1**	**EP**	
L	2.59E+08 ± 1.02E+07^{c}	-1.15^{aA}	-1.71^{bA}	< 0.000001
LF	2.59E+08 ± 1.02E+07^{c}	-4.06^{aC}	-4.11^{bC}	< 0.000001
BC	2.59E+08 ± 1.02E+07^{b}	-3.25^{aB}	-3.38^{aB}	< 0.000001
		< 0.000001	< 0.000001	*p* value
Lactobacillales	**BL**	**T1**	**EP**	
L	3.00E+05 ± 3.55E+04^{a}	1.13^{bB}	0.90^{cA}	< 0.000001
LF	3.00E+05 ± 3.55E+04^{a}	1.35^{bA}	-0.74^{cC}	< 0.000001
BC	3.00E+05 ± 3.55E+04^{a}	-0.30^{cC}	0.12^{bB}	0.000157
		< 0.000001	< 0.000001	*p* value
Bifidobacteriaceae	**BL**	**T1**	**EP**	
L	6.30E+05 ± 3.32E+04^{c}	-2.81^{b}	-2.34^{a}	< 0.000001
LF	6.30E+05 ± 3.32E+04^{b}	-2.06^{a}	-1.93^{a}	0.000023
BC	6.30E+05 ± 3.32E+04^{b}	-3.98^{a}	-2.59^{a}	0.000003
		0.423431	0.093098	*p* value
Enterobacteriaceae	**BL**	**T1**	**EP**	
L	7.37E+05 ± 4.39E+04^{a}	5.16^{cB}	6.14^{bC}	0.000143
LF	7.37E+05 ± 4.39E+04^{a}	8.87^{aA}	8.70^{bA}	0.000065
BC	7.37E+05 ± 4.39E+04^{a}	5.00^{aB}	7.41^{bB}	0.001236
		0.000617	0.000457	*p* value

^{A,B,C}Different capital letters indicate significance difference within a column; ^{a,b,c}Different lower case letters indicate statistical significance within a row according to ANOVA model followed by Tukey's HSD test *(P* < 0.05). MANOVA *p* values are relative to "time effect" on rows and to "matrix effect" on columns. L = milk with Lactose; LF = milk Lactose-Free; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

9.3.1.4. *Metataxonomy of the human colon microbiota before and after in vitro fermentation*

Metataxonomy results of HCM demonstrated that at least two main phyla (*Bacteroidetes* and *Proteobacteria*) were significantly shifted in any samples from the BL to the EP (Table 2). Interestingly, these two are particularly involved in fibrolytic and proteolytic fermentations, respectively.

Table 2. Metataxonomy of Abundances and Changes of Colon Microbiota Selected Taxa*.

OTU ID$^{\#}$	**Relative Quantification (%)**				**Changes as Log$_2$(F/C)**			**ANOVA**
	BL	EP			EP			*p* value
Phylum level	mean	L	LF	BC	L	LF	BC	
Euryarchaeota	0.060	0.124	0.030	0.001	1.05	-1.01	-5.79	0.921606
Bacteria; Other	6.752	0.059	0.285	0.127	-6.84	-4.56	-5.73	0.000413
Actinobacteria	4.956	2.127	0.800	6.315	-1.22	-2.63	0.35	0.629394

Bacteroidetes	35.945	0.385	1.058	9.109	-6.55	-5.09	-1.98	0.028601
Firmicutes	49.499	22.992	55.361	25.576	-1.11	0.16	-0.95	0.548720
Proteobacteria	2.726	74.244	42.429	58.857	4.77	3.96	4.43	0.043499
Verrucomicrobia	0.017	0.016	0.000	0.000	-0.12	0.00	0.00	0.376325
Family level								
Bacteroidaceae	13.241	0.196	0.742	5.814	-6.07	-4.15	-1.18	0.023573
Bifidobacteriaceae	4.938	6.287	2.118	0.796	0.35	-1.22	-2.63	0.023010
Enterobacteriaceae	0.625	71.875	40.229	56.971	6.84	6.00	6.50	0.095932
Clostridiaceae	0.458	6.665	25.089	0.441	3.86	5.77	-0.05	0.048470
Ruminococcaceae	17.338	0.906	10.312	0.300	-4.25	-0.75	-5.85	0.045077
Lachnospiraceae	19.520	2.564	10.429	6.571	-2.92	-0.90	-1.57	0.019588
Peptostreptococcaceae	0.642	1.306	0.251	0.331	1.02	-1.35	-0.95	0.036126
Enterococcaceae	0.240	6.572	6.471	0.202	4.77	4.75	-0.25	0.008922
Lactobacillaceae	0.070	0.117	0.042	0.056	0.74	-0.74	-0.33	0.908709
Leuconostocaceae	0.005	0.019	0.007	0.000	1.86	0.47	0.00	0.578535
Streptococcaceae	0.533	0.922	0.124	0.288	0.79	-2.10	-1.66	0.050969
Species level								
Bacteroides massiliensis	1.699	0.001	0.020	0.859	-10.76	-6.38	-0.98	0.130978
Bacteroides ovatus	0.465	0.141	0.564	0.605	-1.72	0.28	0.38	0.073134
Bacteroides uniformis	2.192	0.003	0.048	0.647	-9.12	-5.49	-1.76	0.041981
Roseburia faecis	4.479	0.066	2.005	0.004	-6.07	-1.16	-10.02	0.102169
Faecalibacterium prausnitzii	8.852	0.196	5.058	0.020	-5.49	-0.81	-8.75	0.004252
Escherichia;Other	0.036	6.325	4.008	52.824	7.43	6.77	10.49	0.576626
Klebsiella;Other	0.134	10.631	1.580	0.380	6.30	3.55	1.50	0.043242
Klebsiella;s__	0.113	32.130	1.296	0.407	8.15	3.51	1.85	0.646024

*Sequencing of each sample was obtained from pooled DNA of two different experiments. The two experiments were performed with two sets of pools of colon microbiotas from three lactose intolerant certificated volunteers; [#] Constructed from Biome files; [§]*Faecalibacterium prausnitzii*
ANOVA for group comparison of BL means and EP values. BL = Baseline; EP = Endpoint; L = milk with Lactose; LF = milk Lactose Free.

Metataxonomy data of HCM at the family level were filtered to discuss those families involved in milk fermentation, and the results demonstrated that some taxa were not affected by the fermentations of both the milk samples, while others were modulated on a time and substrate dependency (Table 2). For example, amongst those that did not significantly change it is of interest to mention the

Lactobacillaceae and the *Streptococcaceae*. Such feature could be attributable to their specialization in metabolization of different dairy sugars.

Among those families that were significantly affected by milk fermentations, *Enterobacteriaceae* were overrepresented at any EP of any sample, with a prominence for L, but not significantly in respect to LF. The culprits of the recorded surges were mostly species of genus *Escherichia* and *Klebsiella*, with the exclusion of pathogenic ones that did not match from the sequencing database. In particular, the increment observed of two *Klebsiella* taxa were averagely double in L than in LF. *Enterobacteriaceae* is avid of any dairy carbohydrate (Hervert et al., 2017) and makes no selective differences. Also, *Bacteroidaceae* were significantly modulated by colonic fermentation, but that of L accounted for the top reduction of about 5 more times lower in respect to the BC. For example, *Bacteroides uniformis* was reduced 9.12 folds after fermentation of L, almost twice stronger than LF. In this situation the results are clearer, evidencing that these important butyrate-producer commensals were more underrepresented when exposed to lactose, as a results of the higher innate symbiosis to the lactose intolerant host ecosystem that makes them unable to face the lactose insult and utilize other sugars. Such more negative effect of L in respect to LF fermentation is confirmed at the species level with the higher depletion of renown health-related taxa, as *Faecalibacterium prausnitzii* (6.7 times more) and *Roseburia faecis* (5.5 times more). Similar trends were also seen in vivo models with lactose intolerant microbiota (Ntemiri et al., 2019; Xue et al., 2020). Still among *Clostridiales*, it is to notion the changes observed in the *Peptostreptococcaceae* family, which includes several pathogens (Milani et al., 2016). From our results this family raised just with the fermentation of milk with lactose.

9.3.2. HEALTHY ADULTS

(Unsubmitted results. Recipient results of an article in preparation)

9.3.2.1.Volatilome analysis

Through SPME GC-MS, among 18 duplicated cases (n = 36), 80 molecules were identified and presented as a quantification heatmap (Figure S2) with more than 80% of similarity with NIST 11 MSMS library (NIST, USA).

A PCA of 11 organic acids distributed cases on the plot, allowing samples to be easily distinguished by colonic fermentation time, discriminating, in addition, L (standard milk) from LF (lactose-free milk) and BC (Figure 3A). From our results, the main descriptor of fermentation with LF was Butanoic acid (by MANOVA approximately 35.80% and of production) (Table S6), similar result to that obtained with the microbiota of lactose-intolerants. The descriptors of L at the EP were principally Pentanoic acid and Propanoic acid, 2-methyl (approx. 52.02% and 65.48% of production, respectively) (Table S6). In particular, L was only responsible for the production of Benzoic acid, methyl ester and of Octanoic acid. Octanoic acid was found to improve the immunological barrier function of the epithelium by inducing the mRNA and protein expression of pBD-1 and pBD-2, representing a novel method to prevent bacterial infections and intestinal disorders in animals and humans (Wang et al., 2018).

A PCA of 21 alcohols separated the BL from substrates fermentation time points, discriminating the BC from the samples L and LF (Figure 3B). The contribution on alcohols production was not discriminated depending on the matrix, except for BC, but it was on a time dependence. The descriptors of L were 2-Octen-1-ol, (E) and 1-Propanol (62.75% and 48.33% of contribution in production, respectively), while LF was described by 3-Buten-1-ol, 3-methyl-, Benzyl Alcohol, Phenethyl Alcohol and Phenol, 4-methyl (66.9% 42.82%, 51.20% and 33.23% of contribution in production, respectively) (Table S6). With the exception of 1-Propanol and Phenol, 4-methyl, all the molecules mentioned above were produced after the fermentation but were absent at the BL (Table S7).

A PCA of 11 other VOCs separated the BL from the fermentation time points of any substrate, discriminating samples based on fermentation time rather than matrix. As for alcohols production, the contribution to other VOC production was not discriminated according to matrix but was found to be time-dependent (Figure 3C). The VOC that defined L was Dimethyl trisulfide (about 67.69%), while LF was mainly described by Indole (about 38.46%) (Table S6). The latter increased throughout

the fermentation (about 33.18% and 54.53% at T1 and EP, respectively), starting from a physiological initial amount (about 12.29%) (Table S7).

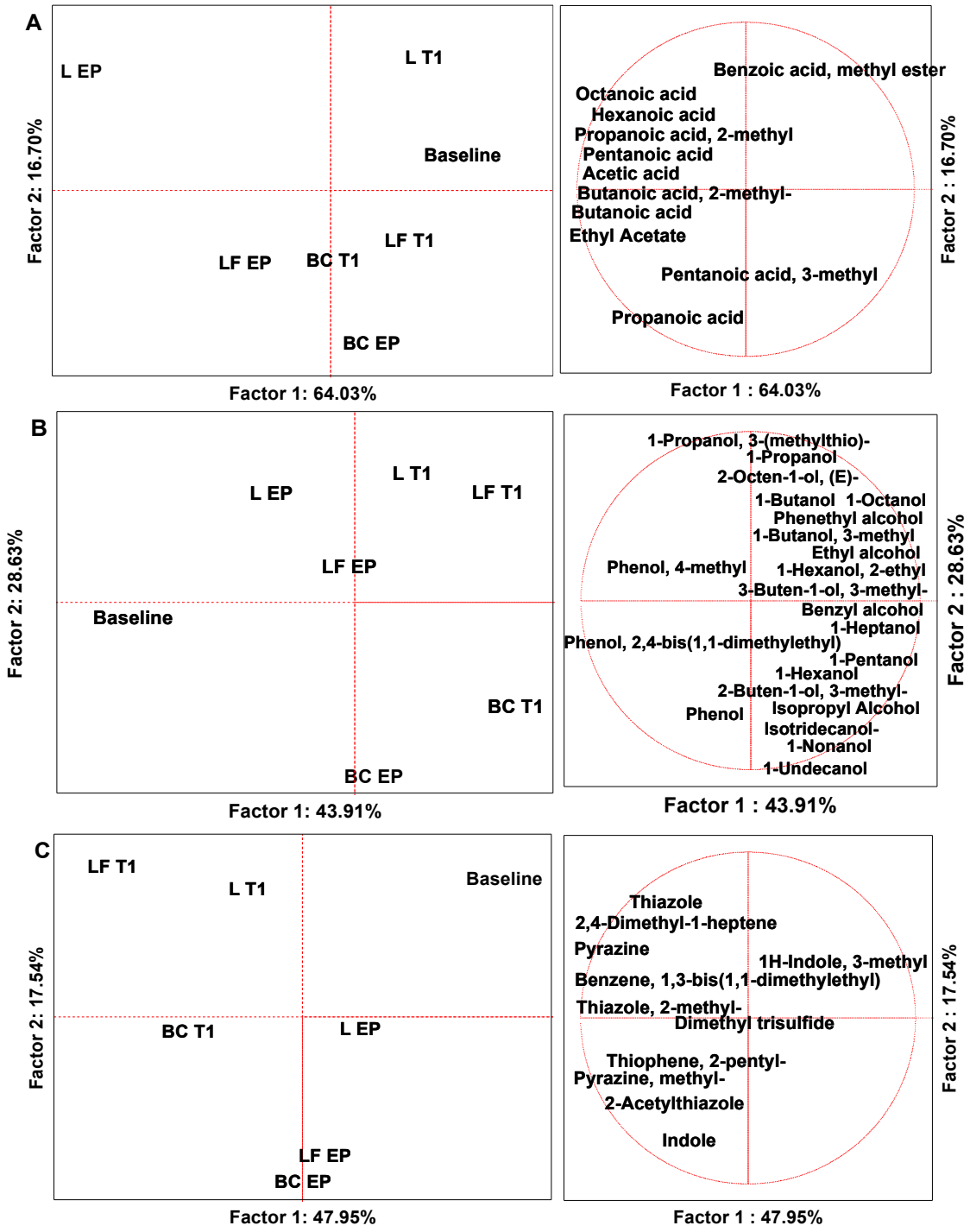


Figure 3. PCA plots of the volatilome sorted by chemical classes, including the biological replicas of L, LF, BC, and the baseline (BL) and different time points (T1 = 18 h and EP = 24 h). A) Acids; B) Alcohols; C) Other VOCs. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. L = milk with Lactose; LF = milk Lactose-Free; BC = Blank Control.

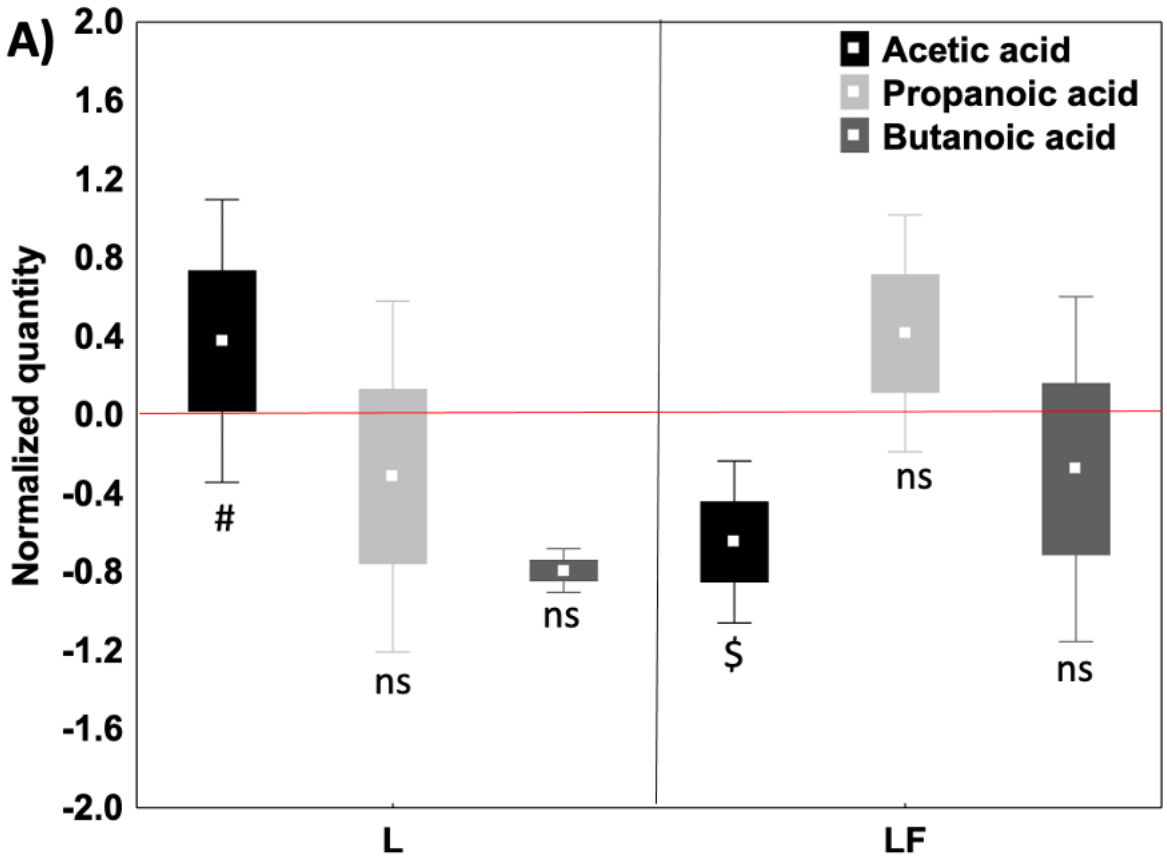
CASE STUDY 6: Lactose-free milk. Healthy adults.

9.3.2.2.Changes in abundance of main microbial VOCs

The three short chain fatty acids (SCFA), namely Acetic, Propanoic, and Butanoic acids, were considered. SCFAs have been shown to have beneficial effects on human health. In fact, these compounds have anti-inflammatory, immunoregulatory and cardiovascular protective activities, among others (Tain et al., 2021; Xiong et al., 2022). The absolute quantifications at the BL (Table S8) were compared to that at the two time points, T1 and EP, and the difference measured and normalized (Figure 4A). Considering the shift of the fermentations with respect to BL, the results showed that both fermentation types tested were unable to produce low-molecular organic acids, except for Acetic acid for L and Propanoic acid for LF.

Although neither of the two milk samples were able to increase SCFA production overall, with the exception mentioned above, it appears that milk containing lactose (L) maintained a ratio of the three compounds closer to the optimum 60:20:20 (Alexandre et al., 2013). Overall, SCFA production in the present study showed similar results as in the previous study with lactose intolerants. Indeed, in both cases, increased Acetic acid production was observed with L, while Propanoic acid and Butanoic acid were reduced. Similarly, in both studies LF causes a greater SCFA unbalance. However, the insult is greater in the present study with the microbiota of healthy subjects because a reduction in two of the three SCFA considered is observed.

CASE STUDY 6: Lactose-free milk. Healthy adults.



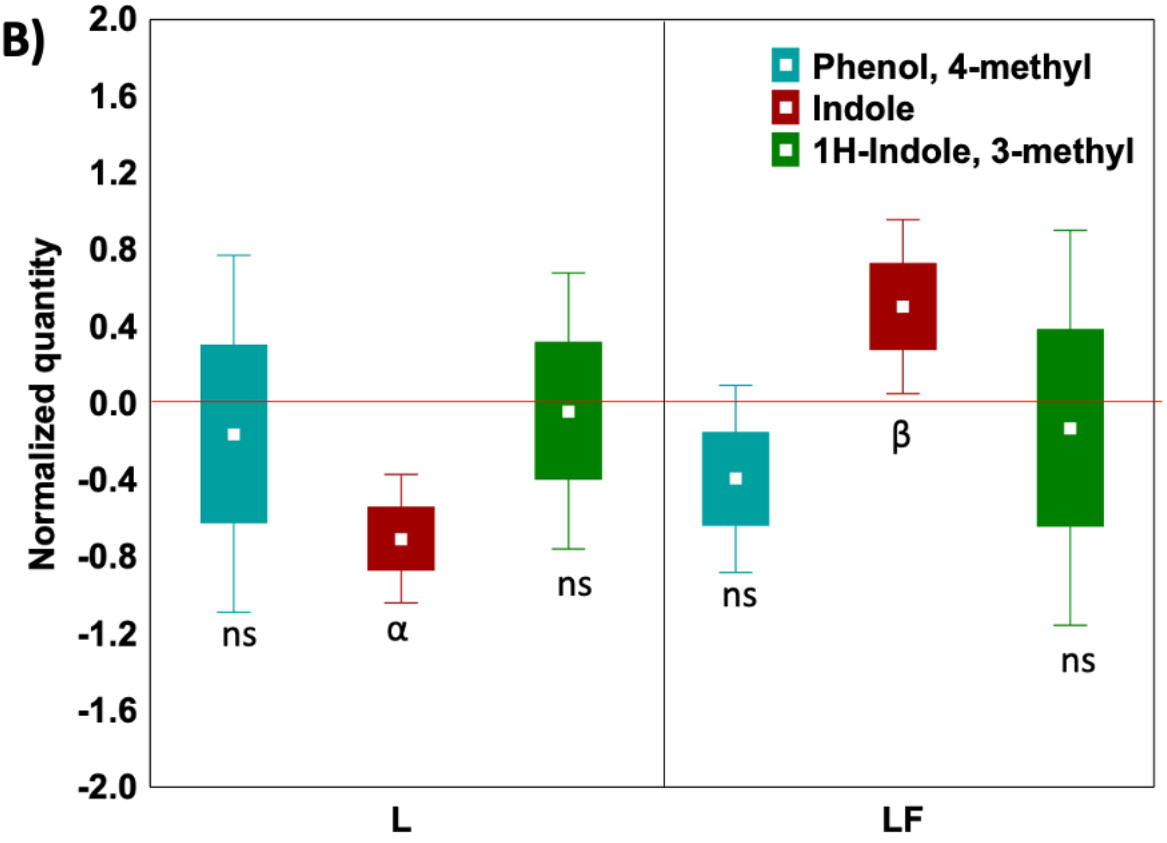


Figure 4. Changes in the abundance of A) beneficial and B) detrimental microbial VOCs in respect to the baseline of in vitro fermentation (red line). Box plots are including all replicas of T1 and EP values. Marker = mean; box = mean ± standard error; whiskers = mean ± standard deviation. Different symbols among a single independent variable indicate significant difference according to MANOVA model followed by post hoc Tukey's HSD test. ns = not significant; L = milk with lactose; LF = milk lactose free.

In contrast to SCFAs, the products of lipid oxidation and amino acid fermentation of the three aromatic amino acids (tyrosine, tryptophan, and phenylalanine) are undesired because they may be toxic and harmful to the host mucosa and compromise colon health. In our results, Phenol, 4-methyl (p-cresol), Indole and 1H-Indole, 3-methyl (skatole) were considered, where the last two are the main dead-end products by intestinal bacteria (Diether et al., 2019). p-Cresol can cause DNA damage and alter the cell cycle by reducing colonocyte proliferation (Bansal et al., 2010). Indole, on the other hand, such as attenuating indicators of inflammation (Ntemiri et al., 2019), becomes toxic to the host when accumulated and transformed in indoxyl sulfate, correlated with renal disease progression and vascular dysfunction in chronic kidney disease (Wang et al., 2020). Lastly, skatole is derived from bacterial decarboxylation of tryptophan, and it has been shown to possess AhR agonis activity in primary human hepatocytes, colon cell lines (Caco2) and intestinal epithelial cells (Diether et al., 2019).

The absolute quantifications at the BL (Table S9) were compared to that at the two time points, T1 and EP, and the difference measured and normalized (Figure 4B). Fermentation of lactose-containing milk (L) did not increase the production of microbial these VOCs. Instead, LF fermentation increased Indole production, in contrast to what was seen with lactose-intolerant donors (Figure 2B). In fact, in the present study LF generated a significant higher amount of Indole in respect to L ($P < 0.05$).

9.3.2.3.Changes in abundance of selected bacterial targets absolutely quantified by qPCR

Considering *Eubacteria*, *Firmicutes*, and *Bacteroidetes* (Table 3), fermentations of both milks decreased their abundances, with LF as the strongest, as already observed with the lactose intolerants. In particular, considering the total *Eubacteria* (Table 3), both milk samples L and LF decreased the abundance in comparison to their own BL, reaching the higher reduction with LF ($P > 0.05$). Similar trend was observed for the blank control (BC).

The quantification of *Firmicutes* phylum (Table 3) appeared reduced as early as after 18 of fermentation, further reducing at the EP ($P < 0.05$). In particular, fermentation of LF was the one that reduced the load of this taxon the most, although there was no significant difference with the reduction recorded with L ($P > 0.05$).

Considering *Bacteroidetes* (Table 3), significant decreases were observed at EP for any sample ($P < 0.05$), with significant differences between L and LF ($P < 0.05$).

Although our results showed a reduction of both *Firmicutes* and *Bacteroidetes* with both milk samples, the reduction of *Firmicutes* with L was lower than that recorded for *Bacteroidetes*. This result is in agreement with a study (Ntemiri et al., 2019) in which the authors observed that in a mouse model of elderly gut microbiota the lactose-containing milk diet sustained a higher *Firmicutes* relative abundance compared to that of *Bacteroidetes*.

Taking the beneficial bacteria into consideration, a decrease of *Lactobacillales* and *Bifidobacteriaceae* abundance was observed only with LF while L supported their growth (Table 3). The genus *Bifidobacterium*, in fact, is a group known to metabolize lactose.

In a study by Vitiello et al. (2019), which investigated the effect of a new probiotic formulation on lactose intolerant people, higher levels of Acetic acid and Propanoic acid, 2-methyl were correlated with increased *Bifidobacterium*. Accordingly, *Bifidobacteriaceae*, which included the *Bifidobacterium* genus, increased only with the fermentation of L, a sample that, as seen above, was described by molecules comprising Acetic acid and Propanoic acid, 2-methyl (Figure 3A).

To observe the shifts during colonic fermentation of the opportunistic part of the microbiota, we have selected the *Enterobacteriaceae* family as they are involved in lactose breakdown (Vipperia et al., 2012). Any substrate tested was able to promote the growth of *Enterobacteriaceae*, without significant differences among L and LF. Probably, the lactose-free milk did not change the ecosystem enough to provide a competitive disadvantage to certain taxa in this family able to metabolize lactose, including *Enterobacter spp.* and *Klebsiella spp.* which are potential pathogens and can cause infections in humans.

Table 3. Enumeration of selected bacterial targets by qPCR.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Eubacteria	**BL**	**T1**	**EP**	
L	2.24E+09 ± 7.00E+07	-0.16^{A}	-0.23	*0.606265*
LF	2.24E+09 ± 7.00E+07^{b}	-1.17^{aB}	-0.59^{a}	*0.002868*
BC	2.24E+09 ± 7.00E+07^{b}	-0.51^{aA}	-0.50^{a}	*0.025652*
		0.004994	*0.559975*	***p*value**
Firmicutes	**BL**	**T1**	**EP**	
L	2.04E+09 ± 1.57E+07^{b}	-0.89^{aB}	-0.94^{aB}	*< 0.000001*
LF	2.04E+09 ± 1.57E+07^{c}	-1.00^{aB}	-1.26^{bB}	*< 0.000001*
BC	2.04E+09 ± 1.57E+07^{b}	-0.42^{aA}	-0.50^{aA}	*0.000455*
		0.000081	*0.0000434*	***p* value**
Bacteroidetes	**BL**	**T1**	**EP**	
L	1.47E+08 ± 1.00E+07^{c}	-0.43^{aB}	-1.14^{bC}	*0.000002*
LF	1.47E+08 ± 1.00E+07^{a}	-0.09^{aA}	-0.70^{bB}	*0.000008*

BC	1.47E+08 ± 1.00E+07^{c}	-0.62^{bB}	-0.11^{aA}	*0.000199*
		0.000680	*0.000028*	***p* value**
Lactobacillales	**BL**	**T1**	**EP**	
L	7.86E+04 ± 4.74E+03^{c}	0.67^{bA}	1.16^{aA}	*< 0.000001*
LF	7.86E+04 ± 4.74E+03^{c}	0.59^{aB}	-0.25^{bB}	*0.000006*
BC	7.86E+04 ± 4.74E+03^{c}	-0.35^{bC}	-0.20^{aB}	*0.000423*
		0.000003	*< 0.000001*	***p* value**
Bifidobacteriaceae	**BL**	**T1**	**EP**	
L	6.15E+05 ± 1.64E+04^{b}	0.49^{a}	0.39^{aA}	*0.001150*
LF	6.15E+05 ± 1.64E+04	1.82	-1.57^{B}	*0.151903*
BC	6.15E+05 ± 1.64E+04^{a}	0.04^{a}	-2.56^{bC}	*0.000033*
		0.261793	*0.001231*	***p* value**
Enterobacteriaceae	**BL**	**T1**	**EP**	
L	2.38E+05 ± 7.60E+03^{ab}	5.76^{b}	7.71^{aA}	*0.000034*
LF	2.38E+05 ± 7.60E+03c	5.59^{b}	7.94^{aA}	*0.000002*
BC	2.38E+05 ± 7.60E+03^{b}	5.93^{a}	2.37^{bB}	*< 0.000001*
		0.760759	*0.000001*	***p* value**

^{A,B,C}Different capital letters indicate significance difference within a column; ^{a,b,c}Different lower case letters indicate statistical significance within a row according to ANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* values are relative to "time effect" on rows and to "matrix effect" on columns. L = milk with Lactose; LF = milk Lactose-Free; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

9.4. Conclusions

This study reports for the first time the effects of lactose-free milk, compared to the control containing lactose, on a batch colonic fermentation model simulating human colonic microbiota from lactose-intolerant and healthy subjects.

The results showed that the fermentation of lactose resulted in an effective insult for the host HCM of lactose-intolerant adults, documented by the depletion of commensals butyrate producers (*Ruminococcaceae* and *Lachnospiraceae*), and commensal fibrolytic *Bacteroidaceae* and by the raise in dysbiotic and diarrhea inducers, either at the phylum and family levels (*Proteobacteria* and *Enterobacteriaceae*) and also by the raise of opportunistic *Peptostreptococcaceae.* The impact of the presence of lactose in the HCM of lactose intolerant adults seems not to affect those bacterial groups more specialized in metabolizing dairy relative sugars, as those are innately adapted to switch their metabolism to different sugars substrates, such as *Lactobacillaceae*, *Streptococcaceae*, and *Bifidobacteriaceae*. In contrast, the presence of lactose seems to affect more that part of the microbiota that is less specialized in the uptake of dairy sugars, and in particular those commensal groups which for that reason may be less competitive in the host ecosystem during life (lack of enzymes for lactose) and consequently silenced the expression of that dedicated metabolic pathways. Considering, the changes in the metabolites production during colonic fermentation, we evidenced

the negative effect of lactose presence towards the HCM of lactose intolerant adults, as the reduction in production of Butanoic acid, possibly linked to the depletion of butyrate-producers taxa.

On the contrary, the absence of lactose generated an insult for the host HCM of healthy adults, demonstrated by the depletion of the beneficial populations of the microbiota, *Lactobacillales* and *Bifidobacteriaceae* and, considering the changes in the detrimental metabolites production during the fermentation, by the production of Indole.

9.5. References

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9.6. Supplementary materials

Table S1. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	Target	Sequence 3'-5'	Bp	MT*	Reference	R^2
Eubacteria	*V3-V4 16S*	Eub518-R: ATTACCGCGGCTGCTGG Eub338-F: ACTCCTACGGGAGGCAG	147	57.6 63.5	Lane et al., 1991	0.996
Firmicutes	*V3-V4 16S*	Firm934-F: GGAGYATGTGGTTTAATT Eub338-R: ACTCCTACGGGAGGCAG	300	60.5 63.5	Guo et al., 2008	0.995
Bacteroidetes	*V3-V4 16S*	Bact934-F: GGARCATGTGGTTTAATT Bact1060-R: AGCTGACGACAACCATG	250	58.9 59.4	Guo et al., 2008	0.995
Lactobacillales	*V3-V4 16S*	F-Lac: GCAGCAGTAGGGAATCT R-Lac: GCATTYCACCGCTACACA	340	59.8 58.3	Walter et al., 2001	0.993
Bifidobacteriaceae	*RecA*	RecA-F: CGTYTCBCAGCCGGAYA RecA-R: CCARVGCRCCGGTCATC	220	60.3 59.2	Masco et al., 2006	0.997
Enterobacteriaceae	*V3-V4 16S*	Enterob-F: TGCCGTAACTTCGGGAG Enterob-R: TCAAGGACCAGTGTTCAG	450	64.2 60.3	Bartosh et al., 2004	0.989

*MT = Melting Temperature

Table S2. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors significant among the food matrices.

VOCs	Matrix				*p value*
	Baseline	BC	L	LF	
Ethyl Acetate	0.00	17.16	31.18	51.66	0.282921

Acetic acid	9.51[b]	17.46[b]	50.10[a]	22.93[b]	0.008120
Propanoic acid	30.53	10.18	40.64	18.65	0.386960
Butanoic acid	2.91[b]	17.86[b]	12.06[b]	67.17[a]	0.012258
Decanoic acid, ethyl ester	0.00	0.00	96.89	3.11	0.185680
Benzoic acid, methyl ester	20.08	20.96	32.07	26.90	0.599947
Pentanoic acid	9.50	0.62	71.78	18.10	0.322207
Hexanoic acid	34.37	0.00	65.63	0.00	0.117278
Heptanoic acid	0.00	38.76	11.41	49.82	0.323839
Octanoic acid	3.56	11.30	52.40	32.74	0.419742
n-Decanoic acid	0.00	19.08	59.81	21.10	0.409691
Ethyl Alcohol	0.00	20.05	28.88	51.07	0.063369
1-Propanol	0.00	36.14	18.56	45.31	0.112796
1-Butanol	0.00	2.08	63.36	34.56	0.257745
Isotridecanol-	0.00	36.76	21.60	41.64	0.803899
1-Hexanol, 2-ethyl-	30.30	4.26	13.84	51.60	0.403219
1-Octanol	3.13	25.02	29.81	42.04	0.634688
Phenylethyl Alcohol	0.00	17.92	38.25	43.82	0.250818
Ethanol, 2,2'-oxybis-	59.58[a]	10.35[b]	12.18[b]	17.89[b]	0.006712
Phenol	8.14[b]	13.71[b]	71.57[a]	6.58[b]	0.009343
Phenol, 4-methyl-	71.41[a]	7.53[b]	12.49[b]	8.57[b]	0.000855
Phenol, 2,4-bis(1,1-dimethylethyl)-	24.75	20.18	28.11	26.96	0.498236
2-Butanone	0.00	36.50	34.16	29.34	0.513432
Pyrazine, 2,5-dimethyl-	0.00	4.17	34.32	61.51	0.083652
Benzene, 1,3-bis(1,1-dimethylethyl)-	12.11	25.35	30.99	31.56	0.093667
2-Acetylthiazole	0.00[b]	24.78[ab]	28.68[a]	46.54[a]	0.018424
1,2,4-Triazol-4-amine, N-(2-thienylmethyl)-	22.73[ab]	4.30[b]	41.98[a]	31.00[ab]	0.049740
Benzenamine, N-ethyl-	26.97	20.93	28.26	23.83	0.646501
Aniline	20.54	22.74	30.53	26.20	0.897295
2-Hexanone	16.19	21.31	35.71	26.78	0.327945
Benzothiazole	21.23	18.64	32.54	27.58	0.560079
Indole	6.36	39.70	38.31	15.63	0.056485
1H-Indole, 3-methyl-	79.53[a]	7.13[b]	6.93[b]	6.41[b]	0.000330

[abc]Different letters indicate statistical significance according to ANOVA model followed by post hoc Tuckey's HSD test ($P < 0.05$); L = milk with Lactose; LF = milk Lactose Free

Table S3. MANOVA categorical descriptors for the volatilome, categorized for the time. % of contribution of VOCs descriptors significant among the time.

VOCs	**Time**			***p value***
	BL	**T1**	**EP**	
Ethyl Acetate	0.00	68.65	31.35	0.159600
Acetic acid	13.62	42.42	43.97	0.600896
Propanoic acid	39.73	17.88	42.39	0.449813
Butanoic acid	4.30	43.11	52.59	0.679499
Decanoic acid, ethyl ester	0.00	20.91	79.09	0.642814
Benzoic acid, methyl ester	27.37	29.89	42.74	0.306360

Pentanoic acid	13.61	27.80	58.59	0.781756
Hexanoic acid	47.89	13.58	38.53	0.839550
Heptanoic acid	0.00	30.79	69.21	0.236620
Octanoic acid	5.25	28.60	66.15	0.366750
n-Decanoic acid	0.00	25.75	74.25	0.279641
Ethyl Alcohol	0.00	45.25	54.75	0.293866
1-Propanol	0.00	44.73	55.27	0.214987
1-Butanol	0.00	26.45	73.55	0.396469
Isotridecanol-	0.00	38.98	61.02	0.586019
1-Hexanol, 2-ethyl-	39.47	37.74	22.78	0.894104
1-Octanol	4.62	32.24	63.13	0.225158
Phenylethyl Alcohol	0.00	39.54	60.46	0.225920
Ethanol, 2,2'-oxybis-	68.85[a]	18.42[b]	12.73[b]	0.001475
Phenol	11.74	37.66	50.60	0.814848
Phenol, 4-methyl-	78.93	8.91	12.16	0.000114
Phenol, 2,4-bis(1,1-dimethylethyl)-	33.04[b]	29.38[a]	37.58[a]	0.375364
2-Butanone	0.00	66.18	33.82	0.066506
Pyrazine, 2,5-dimethyl-	0.00	30.57	69.43	0.089281
Benzene, 1,3-bis(1,1-dimethylethyl)-	17.12[b]	38.17[ab]	44.71[a]	0.036974
2-Acetylthiazole	0.00	44.08	55.92	0.079271
1,2,4-Triazol-4-amine, N-(2-thienylmethyl)-	30.61	33.45	35.93	0.984580
Benzenamine, N-ethyl-	35.65	27.31	37.03	0.199699
Aniline	27.94	27.05	45.01	0.239795
2-Hexanone	22.47	32.06	45.47	0.251088
Benzothiazole	28.79	27.50	43.71	0.264537
Indole	9.25	40.20	50.55	0.325333
1H-Indole, 3-methyl-	85.35[a]	5.91[b]	8.73[b]	0.000001

[abc]Different letters indicate statistical significance according to ANOVA model followed by post hoc Tuckey's HSD test ($P < 0.05$); L = milk with Lactose; LF = milk Lactose Free

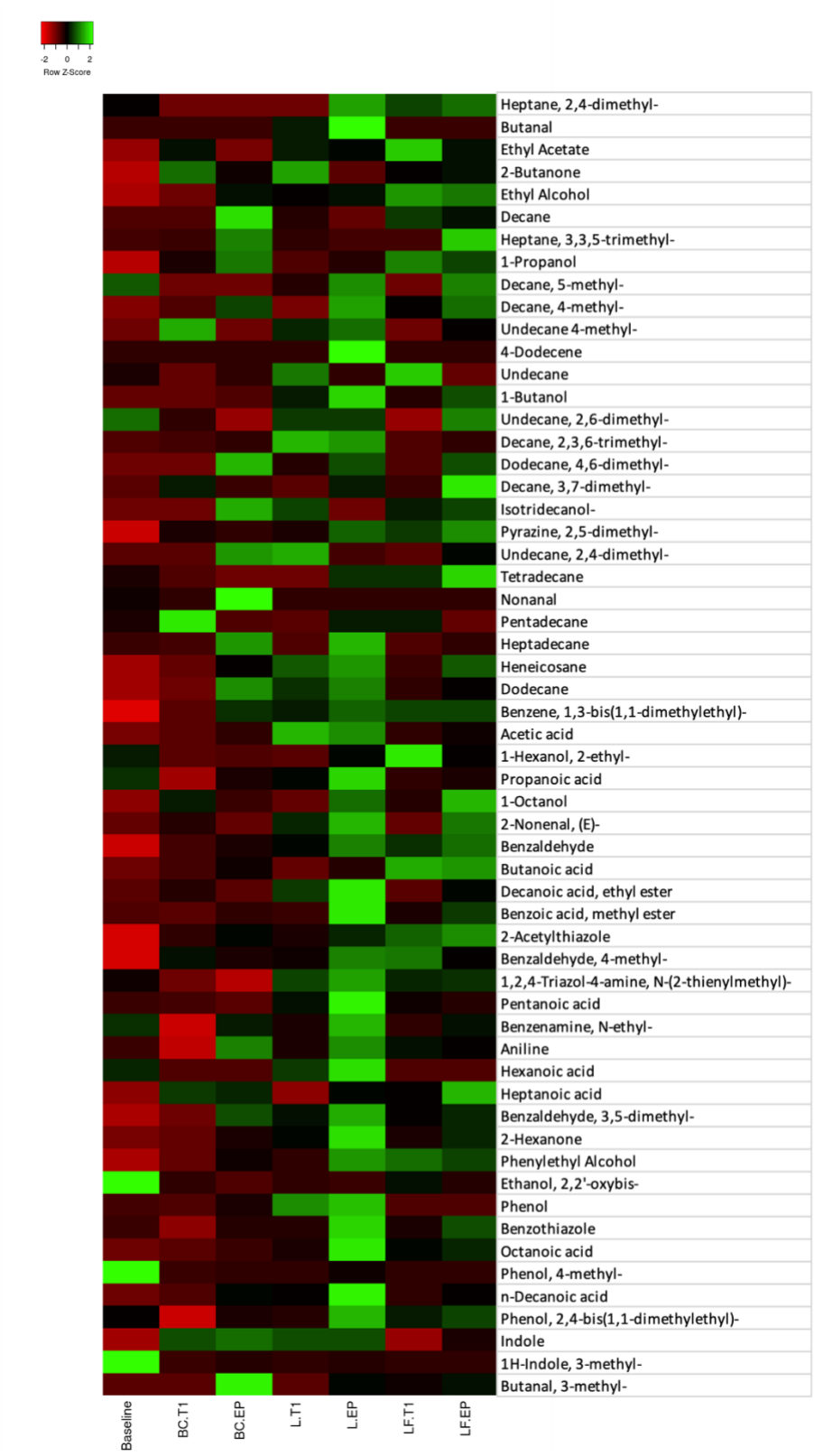

Figure S1. Quantification heatmap of relative abundances of volatile organic compounds (VOCs) sample. BC = Blank Control; T1 = 18 h; EP = 24 h; L = milk with Lactose; LF = milk Lactose Free

Table S4. Baseline values of beneficial VOCs in mM.

VOCs	**Baseline (mM ± SD)**
Acetic acid	286.73 ± 135.40
Propanoic acid	146.64 ± 55.63
Butanoic acid	357.11 ± 118.51

Table S5. Baseline values of detrimental VOCs in mM.

VOCs	**Baseline (mM ± SD)**
Phenol, 4-methyl	135.49 ± 10.20
Indole	40.19 ± 9.10
1H-Indole, 3-methyl	30.01 ± 12.40

Table S6. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors among the food matrices.

Volatile Organic Compounds	% of contribution of VOCs descriptors				MANOVA
	Baseline	**BC**	**L**	**LF**	***P value***
Ethyl Acetate	0.00	22.55	38.48	38.97	0.206198
Acetic acid	29.10	22.12	37.68	11.10	0.328800
Propanoic acid	32.84	27.01	15.69	24.46	0.085876
Propanoic acid, 2-methyl	0.00	16.39	65.48	18.13	0.759150
Butanoic acid	6.71	24.30	33.19	35.80	0.859820
Butanoic acid, 2-methyl-	0.00	22.99	52.95	24.06	0.798437
Benzoic acid, methyl ester	0.00	0.00	100.00	0.00	0.571071
Pentanoic acid	0.00	21.81	52.02	26.18	0.833455
Pentanoic acid, 3-methyl	0.00	100.00	0.00	0.00	0.605610
Hexanoic acid	2.49	4.31	68.21	24.99	0.727496
Octanoic acid	0.00	0.00	100.00	0.00	0.605610
Isopropyl Alcohol	0.00	67.28	13.47	19.25	0.041554
Ethyl alcohol	1.66	30.38	24.46	43.50	0.347908
1-Propanol	0.97^{b}	15.94^{ab}	48.33^{a}	34.77^{ab}	0.038298
1-Butanol	1.27	27.81	34.31	36.61	0.375363
1-Butanol, 3-methyl	0.00	27.90	36.79	35.31	0.377423
1-Pentanol	0.00^{b}	47.22^{a}	19.14^{ab}	33.64^{ab}	0.024339
3-Buten-1-ol, 3-methyl-	0.00	33.08	0.00	66.92	0.752188
2-Buten-1-ol, 3-methyl-	0.00	100.00	0.00	0.00	0.288082
Isotridecanol-	0.00	100.00	0.00	0.00	0.605610
1-Hexanol	0.00	47.40	22.86	29.74	0.081168
1-Heptanol	10.25	37.91	20.94	30.91	0.236838

1-Hexanol, 2-ethyl	11.11	31.51	31.46	25.92	0.498309
1-Octanol	8.32	28.66	31.51	31.50	0.214612
2-Octen-1-ol, (E)-	0.00	7.83	62.75	29.42	0.384360
1-Nonanol	19.33^{c}	37.71^{a}	12.80^{c}	30.16^{bc}	0.004246
1-Propanol, 3-(methylthio)-	0.00^{b}	0.00^{b}	46.21^{a}	53.79^{a}	0.012473
1-Undecanol	0.00^{b}	100.00^{a}	0.00^{b}	0.00^{b}	0.001465
Benzyl alcohol	0.00	36.32	20.86	42.82	0.076428
Phenethyl alcohol	0.00^{b}	24.86^{ab}	23.94^{ab}	51.20^{a}	0.041535
Phenol	2.96	79.74	12.94	4.36	0.627448
Phenol, 4-methyl	21.43	19.03	26.30	33.23	0.931528
Phenol, 2,4-bis(1,1-dimethylethyl)	49.98	18.16	18.18	13.68	0.165495
2,4-Dimethyl-1-heptene	0.00	26.77	25.67	47.56	0.758039
Pyrazine	0.00	31.67	29.44	38.89	0.716937
Thiazole, 2-methyl-	0.00^{a}	15.12^{a}	9.57^{a}	75.32^{b}	0.002113
Thiazole	0.00	24.42	54.18	21.40	0.844314
Pyrazine, methyl-	0.00	56.32	12.63	31.05	0.112721
Dimethyl trisulfide	0.00	5.04	67.69	27.27	0.649985
Benzene, 1,3-bis(1,1-dimethylethyl)	3.92	35.08	26.85	34.15	0.324468
Thiophene, 2-pentyl-	0.00	100.00	0.00	0.00	0.145460
2-Acetylthiazole	0.00	38.59	34.93	26.48	0.150983
Indole	8.54	33.14	19.86	38.46	0.259383
1H-Indole, 3-methyl	45.73	25.70	17.25	11.32	0.239777

^{abc} Different letters indicate statistical significance by Tuckey's HSD test ($P < 0.05$).

Table S7. MANOVA categorical descriptors for the volatilome, categorized for the time. % of contribution of VOCs descriptors among the time.

Volatile Organic Compounds	% of contribution of VOCs descriptors			MANOVA
	0h	**16h**	**24h**	***P value***
Ethyl Acetate	0.00	40.68	59.32	0.091955
Acetic acid	38.11	21.68	40.21	0.517651
Propanoic acid	42.31	27.34	30.35	0.393138
Propanoic acid, 2-methyl	0.00	11.66	88.34	0.426811
Butanoic acid	9.74	20.19	70.07	0.096857
Butanoic acid, 2-methyl-	0.00	15.76	84.24	0.257943
Benzoic acid, methyl ester	0.00	100.00	0.00	0.549449
Pentanoic acid	0.00	15.46	84.54	0.297531
Pentanoic acid, 3-methyl	0.00	0.00	100.00	0.604938
Hexanoic acid	3.69	1.23	95.08	0.326409
Octanoic acid	0.00	0.00	100.00	0.604938
Isopropyl Alcohol	0.00	61.62	38.38	0.531158
Ethyl alcohol	2.46	64.39	33.15	0.055222
1-Propanol	1.44	55.44	43.12	0.285683
1-Butanol	1.89^{b}	63.84^{a}	34.27^{b}	0.006292
1-Butanol, 3-methyl	0.00	59.66	40.34	0.098459

1-Pentanol	0.00	56.45	43.55	0.176883
3-Buten-1-ol, 3-methyl-	0.00	89.02	10.98	0.581776
2-Buten-1-ol, 3-methyl-	0.00	100.00	0.00	0.714481
1-Hexanol	0.00	53.79	46.21	0.211910
1-Heptanol	14.62	50.33	35.05	0.178765
1-Hexanol, 2-ethyl	15.79[b]	52.56[a]	31.65[b]	0.005541
1-Octanol	11.99[b]	50.40[a]	37.61[a]	0.007324
2-Octen-1-ol, (E)-	0.00	80.00	20.00	0.251424
1-Nonanol	26.43	35.83	37.74	0.859861
1-Propanol, 3-(methylthio)-	0.00	43.77	56.23	0.590129
1-Undecanol	0.00	43.67	56.33	0.866596
Benzyl alcohol	0.00	44.91	55.09	0.127237
Phenethyl alcohol	0.00	54.97	45.03	0.256022
Phenol	4.37	11.10	84.53	0.585553
Phenol, 4-methyl	29.04	15.82	55.14	0.090167
Phenol, 2,4-bis(1,1-dimethylethyl)	59.98[a]	11.95[c]	28.07[b]	0.000799
2,4-Dimethyl-1-heptene	0.00[ab]	86.87[a]	13.13[b]	0.030089
Pyrazine	0.00	72.40	27.60	0.110102
Thiazole, 2-methyl-	0.00	54.98	45.02	0.673164
Thiazole	0.00[b]	100.00[a]	0.00[b]	0.002589
Pyrazine, methyl-	2.27	53.15	46.85	0.420185
Dimethyl trisulfide	0.00	19.34	80.66	0.540444
Benzene, 1,3-bis(1,1-dimethylethyl)	5.77[a]	59.50[b]	34.73[c]	0.004898
Thiophene, 2-pentyl-	0.00	90.79	9.21	0.772985
2-Acetylthiazole	0.00	50.72	49.28	0.096672
Indole	12.29	33.18	54.53	0.114502
1H-Indole, 3-methyl	55.83	15.19	29.98	0.092098

[abc] Different letters indicate statistical significance by Tuckey's HSD test ($P < 0.05$). 0h = Baseline; 16h = T1; 24h = EP.

CASE STUDY 6. Lactose-free milk

Figure S2. Quantification heatmap of relative abundances of volatile organic compounds (VOCs) sample. BC = Blank Control; T1 = 18 h; EP = 24 h; L = milk with Lactose; LF = milk Lactose Free

CASE STUDY 6. Lactose-free milk

Table S8. Baseline values of beneficial VOCs in mM.

VOCs	**Baseline (mM ± SD)**
Acetic acid	29.63 ± 13.22
Propanoic acid	19.46 ± 13.85
Butanoic acid	19.09 ± 0.019

Table S9. Baseline values of detrimental VOCs in mM.

VOCs	**Baseline (mM ± SD)**
Phenol, 4-methyl	19.89 ± 9.71
Indole	385.22 ± 140.63
1H-Indole, 3-methyl	375.03 ± 82.13

10. CASE STUDY 7: *In vitro* study of the effect of lactose-free whey on colonic microbiota of healthy and lactose intolerant donors

(Article in preparation)

10.1. Introduction

In the milk processing industry, only 10-20% of milk is recovered as a desired end product while the remaining 80% liquid portion is whey, which is discharged as waste (Birania et al., 2021). To promote sustainable development, there is a need to find sustainable ways to use whey, such as beverage production with or without fermentation.

However, whey has a high lactose content, which may make this product unsuitable for the many consumers who are unable to digest lactose. In fact, lactose intolerance has a high prevalence worldwide, ranging from 57 % to 65 % (Catanzaro et al., 2021). For this reason, public interest is directed toward the development of lactose-free dairy products, whose excellent quality often prompts families to switch to their consumption when only one member is lactose intolerant.

The impact dairy products have on consumer health is, on the one hand, due to their nutritional composition and caloric content but, on the other hand, an important influence is also given by the impact these products have on the gut microbiota.

Animal studies show that milk components (e.g., fat and protein) (Huang et al., 2013; Bai et al., 2016) and dairy derivatives (e.g., casein and whey isolates) (Masarwi et al., 2018) can cause changes in the composition of the gut microbiota, while there is some limited evidence in humans demonstrating the impact of certain dairy groups (e.g., yogurt, acidified milk) on the gut microbiota (Burton et al., 2017). Although there is emerging evidence that overall dietary quality (De Filippo et al., 2010; De Filippis et al., 2016) and individual dietary macronutrients and micronutrients play a role in influencing the composition of the gut microbiota (Lopez-Legarrea et al., 2014; Biesalski et al., 2016), the specific influence of lactose-free dairy products on the composition of the gut microbiota has yet to be elucidated.

Some studies have been concerned with evaluating *in vivo* with clinical trials (Smith et al., 2020) or with mouse models (Garcia et al., 2020) the impact on microbiota and gut health of fermented whey. However, these works used non-delactosed whey.

CASE STUDY 7: Lactose-free whey. Healthy adults

In this study, we evaluated a lactose-free whey fermented by *Lactobacillus bulguaricus* and *Streptococcus thermophilus* and then added of two probiotics, *Bifidobacterium lactis* (BB12) and *Lactobacillus rhamnosus* (LGG) and determined its effect on the gut microbiota of lactose-intolerant and healthy subjects with an *in vitro* colonic model.

10.2. Materials and methods

10.2.1. Human Colon Microbiota (HCM)

HCM was obtained from the stools of two lactose-intolerant volunteers and two healthy volunteers. The volunteers were adults not consuming antibiotics, pre- or probiotic supplements in the 3 months prior to the experiment, normal weight, non-smokers, and with no history of chronic gastrointestinal disorders. The lactose-intolerant volunteers were adults with positivity to lactose breath test. Volunteers were informed of the purposes and procedures of the study and provided their written informed consent, in accordance with the Ethics Committee of the University of Bologna.
Human stools were collected by volunteers in a dedicated sterile container, placed in an anaerobic jar with oxygen catalyst (Oxoid, Thermo Fisher Scientific, USA), transferred to the laboratory, and processed within 2 h. HCM was obtained by homogenizing 2 g of each donation in 36 mL of pre-reduced phosphate buffered saline (PBS) (Wang et al, 2020; Nissen et al, 2022).

10.2.2. Experimental sample

Samples were obtained from the Parmigiano Reggiano cheese production process as part of the Mime4health project. This consisted of a whey sample (SP), a fermented whey sample (SPF) and finally a fermented whey sample with a probiotic mix consisting of nu-trish® BB-12® and nu-trish® LGG® (CHR- Hansen) (SPF+pro).
For the fermentation, the Yoflex - Acidifix® 1.0 culture was used, which is composed of lactose-negative *Lactobacillus bulguaricus* and *Streptococcus thermophilus* that develop exclusively in the presence of sucrose.
In addition, lactase (NOLA® Fit- CHR-Hansen) was added to all whey samples to ensure the absence of lactose in the final product.

10.2.3. In vitro gastric and duodenal digestion

Samples were digested *in vitro*. In a summary, the INFOGEST defined protocol was followed during the digestion process (Minekus et al., 2014). The addition of simulated saliva (containing 75 U/mL -amylase), simulated gastric juice (containing 2000 U/mL pepsin) at an acid pH, and simulated pancreatic juice (containing 10 mM bile and 100 U/mL pancreatin) at a neutral pH resulted in a series

of successive enzymatic treatments during *in vitro* digestion. Following digestion, resultant solutions were frozen at -80 °C to render enzymes inactive for additional *in vitro* colonic fermentation.

10.2.4. Fecal Batch-Culture Fermentation and Samples Collection

Colonic fermentations were conducted for 24 hours in independent vessels using MICODE mimicking the conditions of the proximal colon. Each vessel was aseptically loaded with 10 mL of independent mixtures including fecal slurry (10% w/v of human feces in O_2 reduced PBS) and 1 g of *in vitro* digested whey (SP), 1 g of *in vitro* digested fermented whey (SPF), and 1 g of *in vitro* digested fermented whey with a probiotic mix (SPF+PRO) at a final concentration of 1% (w/v). A fourth vessel was set as blank control (BC) (basal medium and 10% fecal slurry with 1% of digestive enzymes). The protocol just described was applied for fermentations performed with faecal inoculum from healthy donors and for fermentations performed with faecal inoculum from lactose-intolerant donors. Technical replicas of analyses were conducted in triplicates from two independent biological experiments.

10.2.5. Experimental set up and pipeline of activities

Parallel and independent vessels for SP, SPF, SPF+PRO, and BC were run for 24 h after the adaptation of the fecal inoculum, defined as the baseline (BL). Each experiment (one for healthy and one for intolerant donors) consisted of 24 cases (n = 24), including 4 theses (SP, SPF, SPF+PRO, and BC) and 3 time points (BL, 18 h, and 24 h) in duplicate. Samples of the different timepoints were used for qPCR and SPME GC/MS. Technical replicas of analyses were conducted in duplicate for SPME GC/MS (n = 36) and in triplicate for qPCR (n= 54), both from two independent experiments.

10.2.6. Microbiota related analyses

10.2.6.1. DNA extraction

DNA was extracted from the MICODE effluents at each time points (BL, 18 h, and 24 h) using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid purity was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

10.2.6.2. Absolute enumeration of bacterial groups by qPCR

16 different bacterial taxa, namely *Eubacteria*, *Firmicutes*, *Bacteroidetes*, *Lactobacillales*, *Bifidobacteriaceae, Enterobacteriaceae*, *Clostridium* group I, *Clostridium* group IV, *Bacteroides*-

Prevotella-Porphyromonas (BPP) group, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Bifidobacterium longum*, *Escherichia coli* (total), *Escherichia coli* (toxigenic), *Desulfovibrio*. spp., *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*) (Table S1), were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA).

10.2.7. Volatilome analysis

Volatile organic compounds (VOCs) evaluation was performed on a Gas Chromatograph (7890A , Agilent, Santa Clara, CA, USA) Mass Spectrometer (5975, Agilent, USA) operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME (Solid Phase Micro-Extraction) GC-MS protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Nissen et al., 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Preconditioning, absorption, desorption of SPME–GC-MS analysis, and all data processing were carried out according to literature (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021). Molecules Identification was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). The VOCs were then sorted and super-normalized for respective chemical class, i.e., organic acids, alcohols, aldehydes, and ketones (Nissen et al., 2021a). In samples at BL the main microbial metabolites related to fermentation of foods were absolutely quantified in mM by SPME GC-MS and an internal standard (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021).

10.2.8. Data processing and statistical analysis

For metabolomics the VOCs were analyzed differently: i) the volatilome was relatively quantified (Peak Area %), sorted and super-normalized for main chemical classes, then computed for Principal Component Analysis (PCA) and Multivariate ANOVA (MANOVA) ($P < 0.05$) to address specific contributes by categorical predictors; ii) six main VOCs were absolutely quantified and independently normalized and their BL values were subtracted from T1 and EP values, then represented as box plots, including *post hoc* Tukey HSD test ($P < 0.05$). For microbiomics MANOVA ($P < 0.05$) model was used to study the shifts in abundance of qPCR values, as $Log_2(F/C)$ (Love et al., 2014) and compared by *post hoc* Tukey HSD test ($P < 0.05$).

10.3. Results and discussion

10.3.1. LACTOSE-INTOLERANTS

10.3.1.1. Volatilome Analysis throught SPME GC/MS

Though SPME GC-MS, among 9 duplicated cases (n = 18), 60 molecules were identified with more than 80% of similarly with NIST 11 MSMS library and the NIST MS Search program 1.0 (NIST, Gaithersburg, MD, USA). The VOCs were then sorted and super-normalized for respective chemical identity, i.e., organic acids, alcohols, aldehydes, and ketones.

A PCA of 7 organic acids distributed the cases over the plot, with no clear discrimination of samples, except for SP, which was more discriminated from the others (Figure 1A). From our results, the main descriptors of SP fermentation were Propanoic acid and Acetic acid (according to MANOVA, approximately 51.28, 34.42% of the production, respectively) (Table S2). In addition, SP was responsible for the higher production of Pentanoic acid, 3-methyl- (77.05% of contribution in production). SP and SPF+PRO were almost equally described by Pentanoic acid (from MANOVA, 34.13 and 34.73% of the production, respectively).

A PCA of 16 alcohols distributed cases on the plot, separating the blank from time points of fermentation of the substrates (Figure 1B). Among fermentation of the substrates, the most discriminated was SP, whose descriptors were Ethyl alcohol, 1-Propanol, 3-Heptanol, and 1-Nonanol (39.78, 45.45, 41.35, and 38.18% of contribution in production, respectively) (Table S2). In particular, Ethyl alcohol was produced mainly after 24 hours of fermentation (Table S6). On the contrary, the main descriptor of fermentation with SPF+PRO was 1-Butanol (from MANOVA, 31.64% of the production) (Table S2).

A PCA of 11 aldehydes distributed cases on the plot, separating the BL from time points of fermentations of the samples (Figure 1C). Samples were not discriminated from each other, but they were discriminated from the blank. Any fermentation with the substrates were described by 2-Nonenal, (E)- but the largest producer was SPF (from MANOVA, 30.58% of contribution in production) (Table S2). 2-Nonenal, (E)- is derived from lipid oxidation of food and it was reported to limit the growth of several intestinal pathogens at very low concentration (Cho et al., 2004). Most of the aldehydes (6 of 11) found were baseline descriptors. These included Butanal, a common aldehyde in the gut that is formed by the bacterial degradation of leucine. High levels of this metabolite have been associated with inflammation and cancer (Rondanelli et al., 2019).

CASE STUDY 7: Lactose-free whey. Healthy adults

A PCA of 9 ketones distributed cases on the plot, separating the blank from time points of fermentations of the samples (Figure 1D). Ketones could be produced by the oxidation of polyunsaturated fatty acids, microbial metabolism, and AA degradation (Peng et al., 2022). From the results, all fermentations with substrates were described by 2,3-Pentanedione, among which the largest producer was SPF, followed by SPF+PRO (41.93 and 30.93% production, respectively) suggesting that whey fermentation played a key role in the formation of this compound during colonic fermentation.

CASE STUDY 7: Lactose-free whey. Healthy adults

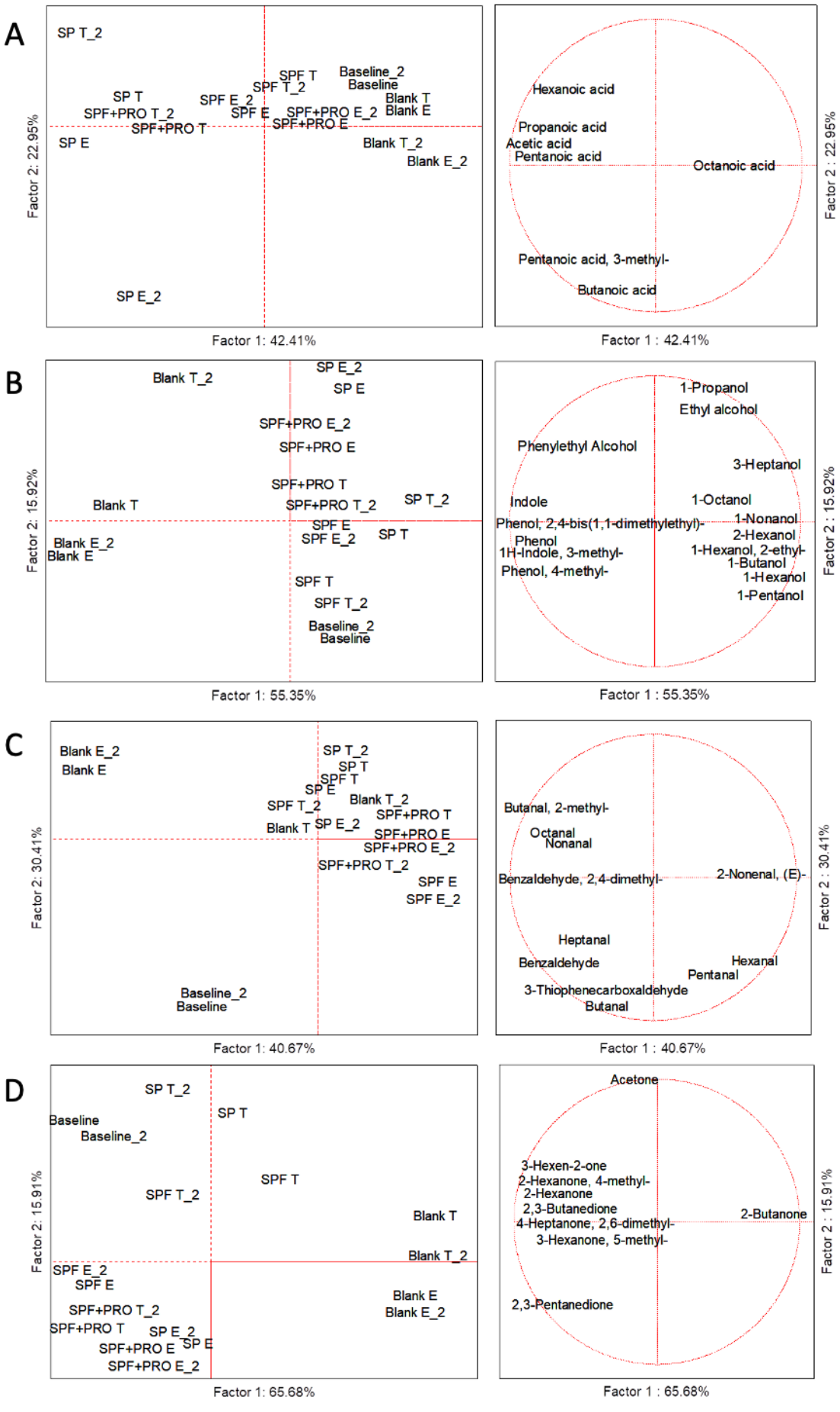

Figure 1. PCAs of the volatilome sorted by chemical classes, including the biological replicas of SP, SPF, SPF+PRO, Blank, and the baseline (BL) and different time points (T = 18 h and EP = 24 h). A) Acids; B) Alcohols; C) Aldehydes; D) Ketones. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of the respective group of cases.

10.3.1.2. Changes in abundance of main microbial VOCs

Three short chain acids were considered, namely Acetic, Propanoic, and Butanoic acids. Previous studies have reported that the production of these three compounds mainly depends on carbohydrate fermentation. However, protein and amino acids fermentation also play an important role in this pool of SCFAs (Sánchez-Moya et al., 2017). SCFAs are important mediators of the interaction between gut microbes and hosts. These bacterial metabolites have been associated with multiple biological activities, such as the regulation of energy homeostasis, anti-inflammatory activity and satiety (Sánchez-Moya et al., 2017). An antimicrobial function has also been associated with SCFAs causing a decrease in the colonic pH inhibiting the growth of some potential pathogens (Sánchez-Moya et al., 2017).

The absolute quantifications at the BL (Table S4) were compared to that of the two time points, T and EP, and the difference measured and normalized (Figure 2A). Considering the shift in fermentations from the BL, the results showed that all samples were able to produce SCFA, with the best result achieved by SP.

In particular, SP produced approximately twice as much Acetic acid as SPF and SPF+PRO while there was no significant difference in Butanoic acid production ($P > 0.05$). Butanoic acid is the preferred fuel of colonocytes and is extensively oxidised by the intestinal epithelium to improve gut health through various local effects and it is mainly produced by *Firmicutes* (Sales et al., 2022). Acetic acid and Propanoic acid are also utilised but are oxidised to a lesser extent than butyrate (Sales et al., 2022). Acetic acid is produced mainly by *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, and *Enterobacteria*.

Production of Acetic, Propanoic, and Butanoic acids occurs in an approximate molar ratio of 60:20:20, respectively, although factors such as bacterial population (type and abundance), diet and intestinal transit time may influence SCFA production.

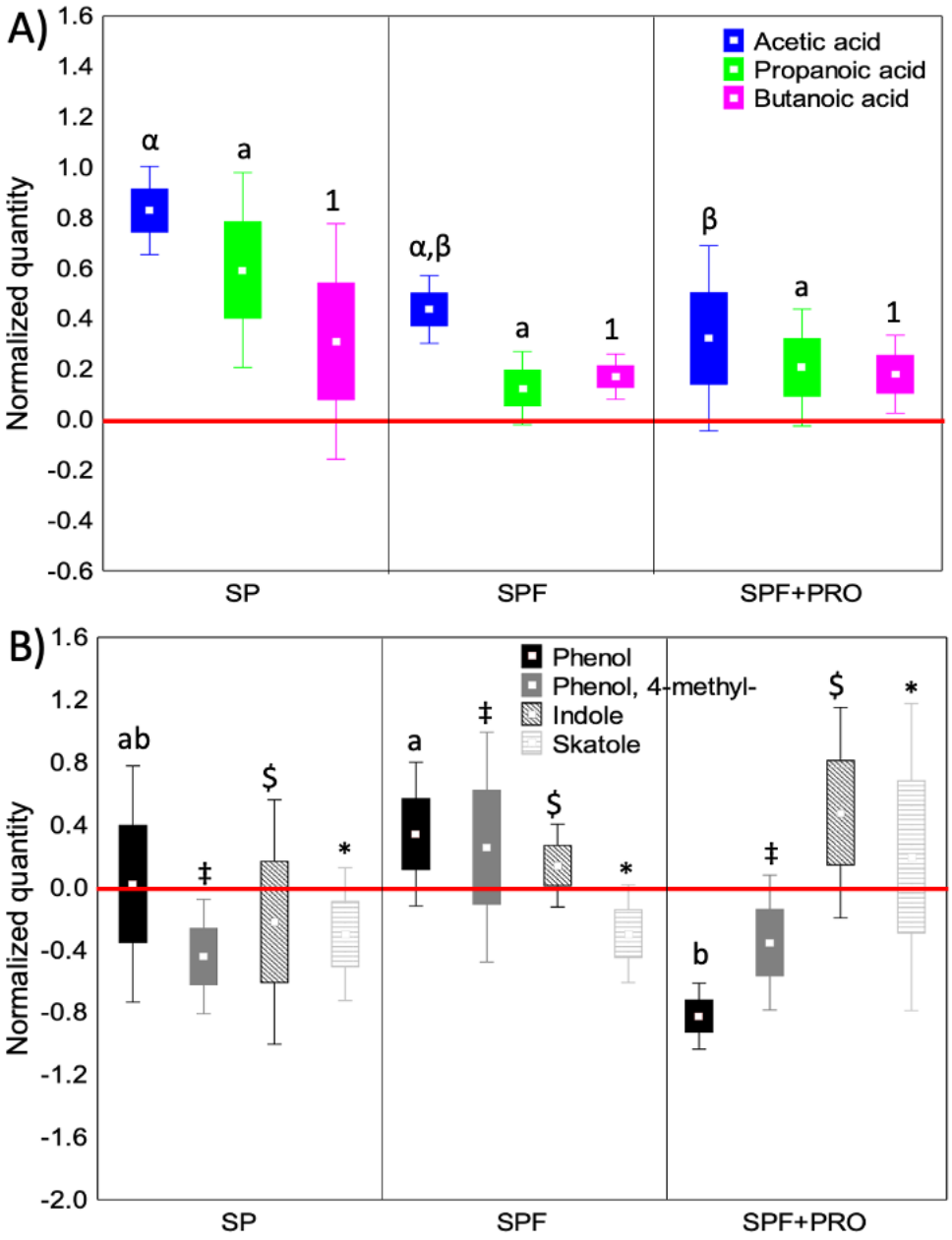

Figure 2. Changes in the abundance of A) beneficial and B) detrimental microbial VOCs in respect to the baseline of in vitro fermentation (red line). Box plots are including all replicas of T1 and EP values. Marker = mean; box = mean ± standard error; whiskers = mean ± standard deviation. Different symbols among a single independent variable indicate significant difference according to MANOVA model followed by post hoc Tukey's HSD test.

In contrast to SCFAs, lipid oxidation and aromatic amino-acids fermentation (mainly of Tyrosine, Triptophan, Phenilalanine) are responsible for the production of VOCs that are potentially detrimental and toxic for the host mucosa. Whey proteins from cow, sheep and goat have shown to be rich in these aromatic amino acids (Sánchez-Moya et al., 2020). From our results, the samples did not perform similarly (Figure 2B).

Starting from the physiological concentrations of these VOCs at baseline (Table S5), the best result was obtained with SP fermentation. In this case, 3 of the 4 metabolites considered were reduced. These included Skatole, a toxic product of bacterial decarboxylation of tryptophan by *Bacteroides spp*. and *Clostridium spp.* that affects the mucosa and causes the production of inflammatory cytokines (Roager and Licht, 2018). Apart from SPF+PRO, all samples were able to reduce Skatole. As already seen for the beneficial SCFA, also for the harmful compounds, the best sample seems to be the SP, which reduced most of the detrimental VOCs.

10.3.1.3. Changes in selected fecal bacterial populations measured with qPCR

10.3.1.3.1. Shift in taxa relative to the core microbiota

Considering the total *Eubacteria* (Table 1), compared to the abundances at the BL and apart from the blank control (BC), at the EP all samples, but SPF+pro, showed statistically significant increases ($P < 0.05$). However, there were no significant differences between SP and SPF ($P > 0.05$).

Taking into consideration the two main phyla of the human colonic microbiota, *Firmicutes* and *Bacteroidetes*, the trends during colonic fermentation were opposite. In fact, while there was an increase in the *Firmicutes* phylum with all whey samples, the *Bacteroidetes* phylum showed a decrease with respect to BL ($P < 0.05$), although without significant differences between samples ($P > 0.05$).

Within the phylum *Bacteroidetes*, *Bacteroides* has been associated with a low-fat or low-carbohydrate diet, as it is a bacterium that is particularly reactive to calorie intake (Santos-Marcos et al., 2019).

Table 1. Quantification of Eubacteria, *Bacteroidetes*, and *Firmicutes*.

qPCR Target	**Cells/mL**	**Log$_2$(F/C)**		**MANOVA**
Eubacteria	**BL**	**T1**	**EP**	
SP	6.30E+09 ± 2.60E+09^{b}	2.96^{a}	2.72^{abAB}	*0.034634*
SPF	6.30E+09 ± 2.60E+09^{b}	2.86^{a}	2.88^{aA}	*0.027565*
SPF+pro	6.30E+09 ± 2.60E+09	2.02	1.69^{B}	*0.056976*
BC	6.30E+09 ± 2.60E+09^{b}	1.90^{a}	2.28^{aAB}	*0.000108*
		0.323586	*0.033114*	***p* value**
Firmicutes	**BL**	**T1**	**EP**	
SP	1.66E+09 ± 1.73E+09c	0.84^{aA}	0.61^{bA}	*<0.000001*
SPF	1.66E+09 ± 1.73E+09	0.20^{B}	0.17^{BC}	*0.081131*
SPF+pro	1.66E+09 ± 1.73E+09	0.23^{B}	0.20^{B}	*0.0374953*
BC	1.66E+09 ± 1.73E+09a	-1.48^{bAB}	-1.55^{cAC}	*<0.000001*
		0.001656	*0.001045*	***p* value**
Bacteroidetes	**BL**	**T1**	**EP**	
SP	4.41E+07 ± 6.67E+06^{a}	-1.72^{bA}	-2.46^{cA}	*<0.000001*
SPF	4.41E+07 ± 6.67E+06^{a}	-2.31^{bB}	-2.84^{cA}	*<0.000001*

SPF+pro	4.41E+07 ± 6.67E+06[a]	-2.86[bC]	-2.97[bAB]	*<0.000001*
BC	4.41E+07 ± 6.67E+06[a]	-3.18[bC]	-3.44[cAB]	*<0.000001*
		<0.000001	*0.000632*	***p* value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test (P < 0.05). MANOVA P value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SP = unfermented whey; SPF = fermented whey; SPF+PRO = fermented whey added with probiotics; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

10.3.1.3.2. Shift in commensal and beneficial taxa

Among the beneficial bacteria considered (Table 2), *Lactobacillales* was significantly reduced with all samples ($p < 0.05$). Among them, however, SP had the least impact on *Lactobacillales* abundance. Similarly, while SPF and SPF+pro reduced the abundance of *Bifidobacteriaceae*, SP was the only sample that was able to promote their growth, which was also observed for *B. longum*.

Within the order *Clostridiales*, we considered *Clostridium group IV*, comprising butyrate-producing bacteria such as *Fecalibacterium prausnitzii*. From our results, the *Clostridium group IV* was significantly reduced after any fermentation compared to BL ($p < 0.05$).

Table 2. Quantification of commensal beneficial taxa.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Lactobacillales	**BL**	**T1**	**EP**	
SP	3.51E+05 ± 1.30E+05[a]	-0.26[b]	-0.29[bAB]	*<0.000001*
SPF	3.51E+05 ± 1.30E+05	-1.06	-1.18[AB]	*0.150219*
SPF+pro	3.51E+05 ± 1.30E+05[a]	-0.83[b]	-1.78[cB]	*0.000028*
BC	3.51E+05 ± 1.30E+05[a]	-0.93[b]	-1.81[cAB]	*<0.000001*
		0.844627	*0.016896*	***p* value**
Bifidobacteriaceae	**BL**	**T1**	**EP**	
SP	3.64E+06 ± 2.08E+06[c]	0.37[aA]	0.20[b]	*<0.000001*
SPF	3.64E+06 ± 2.08E+06	-0.40[AB]	-0.56	*0.051551*
SPF+pro	3.64E+06 ± 2.08E+06	-0.30[A]	-0.42	*0.905850*
BC	3.64E+06 ± 2.08E+06[a]	-2.33[cB]	-1.58[b]	*<0.000001*
		0.010216	*0.209110*	***p* value**
***Clostridium* Group IV**	**BL**	**T1**	**EP**	
SP	7.20E+08 ± 2.29E+08[a]	-1.06[bA]	-1.36[cA]	*<0.000001*
SPF	7.20E+08 ± 2.29E+08[a]	-2.87[cB]	-2.70[bB]	*<0.000001*
SPF+pro	7.20E+08 ± 2.29E+08[a]	-2.65[bB]	-2.71[cB]	*<0.000001*
BC	7.20E+08 ± 2.29E+08[a]	-4.91[cC]	-4.40[bB]	*<0.000001*
		<0.000001	*<0.000001*	**P value**
B. longum	**BL**	**T1**	**EP**	
SP	1.38E+06 ± 5.53E+05[c]	0.98[a]	0.62[b]	*<0.000001*
SPF	1.38E+06 ± 5.53E+05	0.52	-0.29	*0.089482*
SPF+pro	1.38E+06 ± 5.53E+05	0.21	-0.49	*0.165284*
BC	1.38E+06 ± 5.53E+05[a]	-1.09[b]	-1.77[c]	*<0.000001*

		0.128477	*0.402009*	**P value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test (P < 0.05). MANOVA P value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SP = unfermented whey; SPF = fermented whey; SPF+PRO = fermented whey added with probiotics; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

10.3.1.3.3. Shift in commensal and opportunistic taxa

We have chosen particular taxa that have strong proteolysis activity in order to assess the shifts during colonic fermentation of a portion of the opportunistic part of the microbiota (Table 3).

Any substrate tested was able to support the growth of *Enterobacteriaceae* from the BL to EP, although SPF and SPF+PRO did not significantly ($p > 0.05$).

Within the *Enterobacteriaceae* family, the same trend was observed. *E. coli*, in fact, increased significantly with all samples compared to BL ($p < 0.05$), although there were no significant differences between samples ($p > 0.05$).

Considering *Clostridium I group*, the trend was different among the samples. In fact, while there was a decrease with both SP and SPF+pro, SPF was the only one causing an increase.

Table 3. Quantification of commensal opportunistic taxa.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Enterobacteriaceae	**BL**	**T1**	**EP**	
SP	2.05E+05 ± 1.61E+04^{b}	8.49^{a}	8.40^{aB}	*0.025153*
SPF	2.05E+05 ± 1.61E+04^{ab}	8.89^{a}	5.80^{bB}	*0.016558*
SPF+pro	2.05E+05 ± 1.61E+04	7.46	5.99^{B}	*0.182009*
BC	2.05E+05 ± 1.61E+04^{b}	8.61^{b}	10.79^{aA}	*0.000001*
		0.078491	*<0.000001*	***p* value**
***Clostridium* Group I**	**BL**	**T1**	**EP**	
SP	1.69E+06 ± 8.29E+05^{a}	-3.71^{bA}	-7.36^{bA}	*<0.000001*
SPF	1.69E+06 ± 8.29E+05	5.28^{AB}	6.32^{B}	*0.064808*
SPF+pro	1.69E+06 ± 8.29E+05	1.58^{B}	-0.79^{A}	*0.080285*
BC	1.69E+06 ± 8.29E+05^{b}	2.19^{bA}	5.31^{aB}	*0.000224*
		0.017372	*0.023211*	***p* value**
Escherichia coli* (total)	**BL**	**T1**	**EP**	
SP	2.82E+04 ± 6.86E+05^{c}	5.32^{a}	5.00^{bB}	*<0.000001*
SPF	2.82E+04 ± 6.86E+05^{b}	4.75^{a}	4.94^{aAB}	*0.001164*
SPF+pro	2.82E+04 ± 6.86E+05^{b}	4.85^{a}	4.95^{aAB}	*0.000051*
BC	2.82E+04 ± 6.86E+05^{c}	10.84^{a}	10.71^{bA}	*<0.000001*
		0.074512	*0.016200*	***p* value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test (P < 0.05). MANOVA P value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SP = unfermented whey; SPF = fermented whey; SPF+PRO = fermented whey added with probiotics; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

10.3.2. HEALTHY ADULTS

10.3.2.1. Volatilome Analysis throught SPME GC/MS

Through SPME GC-MS, among 9 duplicated cases (n = 18), 100 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). The VOCS were then sorted and super-normalized for respective chemical identity, i.e., organic acids, alcohols, aldehydes, and ketones.

A PCA of 8 organic acids distributed the cases over the plot, discriminating BL and SPF from the others (Figure 3A). From our results, the main descriptors of SPF fermentation were Propanoic acid, Hexanoic acid and Pentanoic acid, 3-methyl (according to MANOVA, approximately 55.41, 51.75 and 52.64% of the production, respectively) (Table S6). Fermentation processes of food proteins, in which whey is rich, have been shown to play a small role in the production of SCFA, mainly in the form of Acetic and Propanoic acid (Markowiak-Kopec´ et al., 2020). In agreement with our results, Smith et al. (2020) observed an increase in propionate concentrations following consumption of fermented whey. Hexanoic acid could result from the conversion by the gut microbiota of amino acids such as valine, leucine and isoleucine from the anaerobic breakdown of proteins (Markowiak-Kopec´ et al., 2020).

A PCA of 17 alcohols distributed cases on the plot, separating the BL from time points of fermentation of the substrates and discriminating the BC from the samples (Figure 3B). The contribution on alcohols production was not clearly discriminated for SP and SPF but it was for SPF+PRO in respect to the other samples. The descriptors of SPF+PRO were 1-Hexanol, 1-Hexanol, 2-ethyl- and 1-Pentanol (34.21, 33.65 and 36.48% of contribution in production, respectively) (Table S6). Considering which type of whey was better for healthy subjects in terms of detrimental compounds, from the volatilome results the whey without added probiotics (SP and SPF) produced Phenol. In particular, according to MANOVA, SP was responsible for 51.62% of Phenol production while, by adding probiotics (SPF+PRO), the production of this detrimental compound was lowered to 8.81%. Phenol is derived from proteolytic fermentation and has been shown to reduce intestinal epithelial barrier function *in vitro* (Wang et al., 2020).

A PCA of 12 aldehydes distributed cases on the plot, separating the BL from time points of fermentations of the samples (Figure 3C). Also, samples were not discriminated from each other, although they were discriminated from the blank. Whey samples did not have any specific descriptor, but all were described by Benzaldehyde, Propanal, Benzaldehyde, 4-methyl- and Benzaldehyde, 3,5-

ethyl-. However, SPF was responsible for the higher production of Benzaldehyde (from MANOVA, 34.97% of contribution in production), an aromatic compound with antimicrobial activity (Wang et al., 2019), followed by SP and SPF+PRO (20.38 and 20.02% of contribution in production, respectively) (Table S6).

A PCA of 14 ketones distributed cases on the plot, separating the BL from time points of fermentations of the samples and SPF+PRO from the others (Figure 3D). SPF and SP were not discriminated from the blank. Descriptors of fermentation with SPF+PRO were Acetone and Mercaptoacetone, a thiolic ketone. The latter was an exclusive descriptor of fermentation with SPF+PRO (Table S6).

CASE STUDY 7: Lactose-free whey. Healthy adults

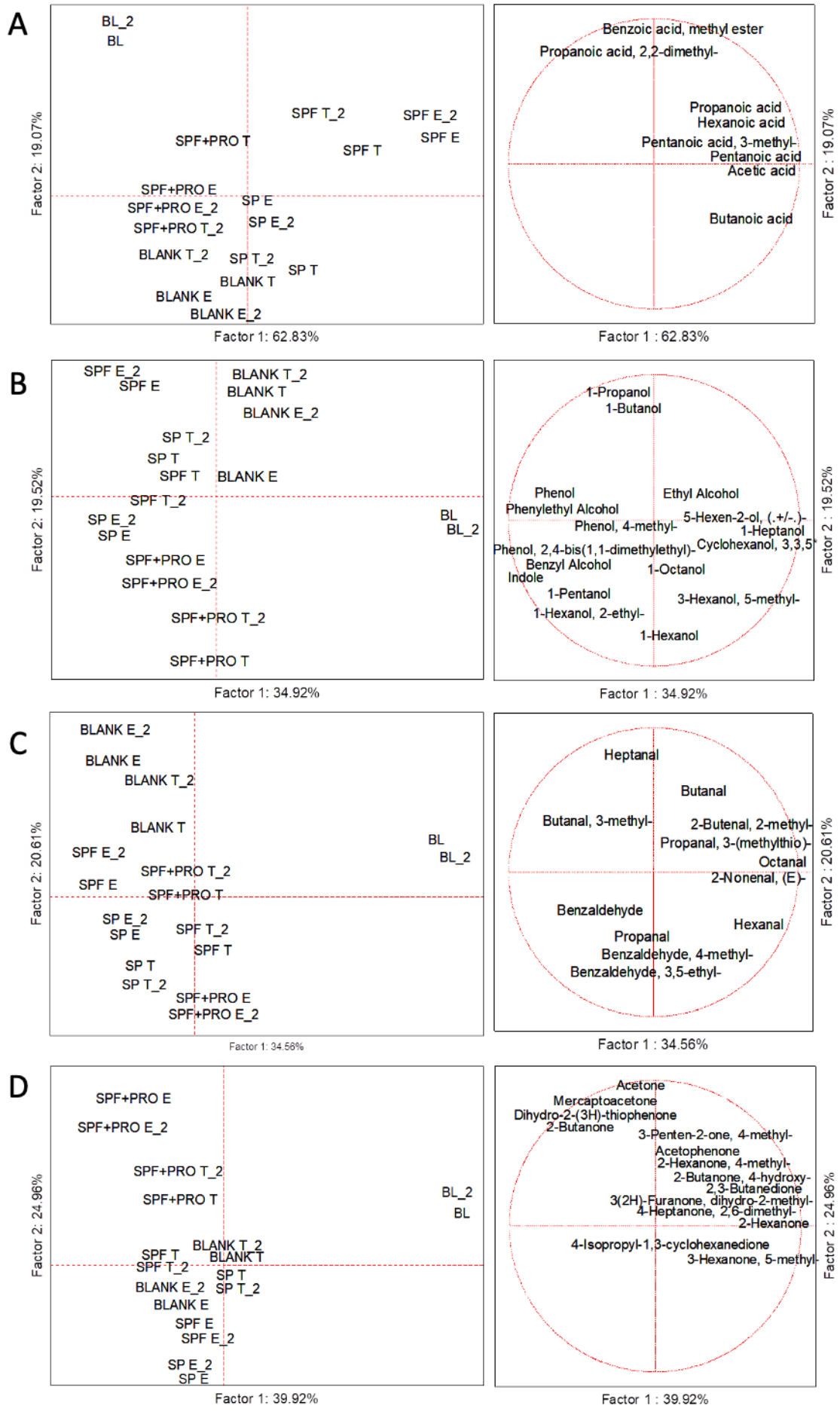

Figure 3. PCAs of the volatilome sorted by chemical classes, including the biological replicas of SP, SPF, SPF+PRO, Blank, and the baseline (BL) and different time points (T = 18 h and EP = 24 h). A) Acids; B) Alcohols; C) Aldehydes; D) Ketones. Left side diagrams are for PCAs of cases; right side diagrams are for PCAs of variables. Variables with different colors are the main descriptors of the respective group of cases.

10.3.2.2. Changes in abundance of main microbial VOCs

The absolute quantifications at the BL (Table S4) were compared to that at the two time points, T and EP, and the difference measured and normalized (Figure 4A).

Considering the shift of the fermentations with respect to BL, the results showed that all samples were able to produce low-molecular organic acids, with the best performance with SPF, followed by SP. In accordance with the results obtained by Sanchez-Moya et al. (2017), the rank of SCFAs after 24 h colonic fermentation was "Acetate > Propionate > Butyrate" acids for the best performing SPF. In particular, SPF was able to produce almost the double of Acetic and Butanoic acid and almost 4 times more Propanoic acid than SP. Possibly, fermentation with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* caused the proteins to be broken down into peptides and amino acids, which were more readily available for fermentation by the gut microbes resulting in the production of SCFAs. In contrast, SPF+PRO did not produce many SCFAs compared to BL.

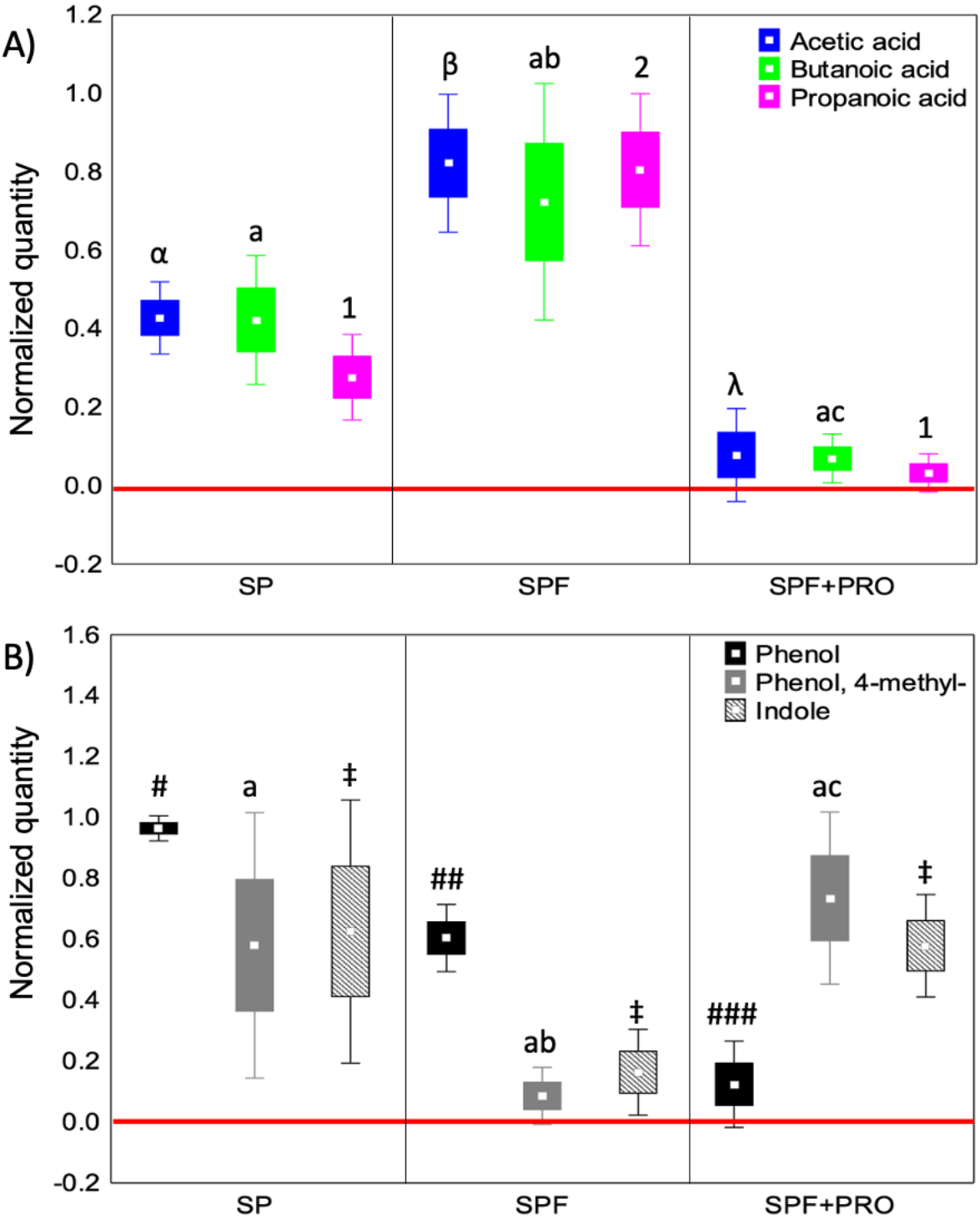

Figure 4. Changes in the abundance of A) beneficial and B) detrimental microbial VOCs in respect to the baseline of in vitro fermentation (red line). Box plots are including all replicas of T1 and EP values. Marker = mean; box = mean ± standard error; whiskers = mean ± standard deviation. Different symbols among a single independent variable indicate significant difference according to MANOVA model followed by post hoc Tukey's HSD test.

In contrast to what has been seen with lactose-intolerant donors, fermentation of any kind of whey has produced detrimental VOCs. In particular, starting from physiological concentrations of these VOCs at the baseline (Table S5), the sample that generated the highest amount was the non-fermented

control SP, followed by SPF+PRO. In respect to this control, SPF produced less amounts of any compounds, reducing for example Phenol by almost twice ($p < 0.05$).

Phenol and p-Cresol have been shown to damage epithelial barrier function *in vitro* and can be potentially carcinogenic (Wang et al., 2020).

Alike the trend of results of beneficial VOCs, SPF was the best sample that produced the least amount of detrimental VOCs (Figure 4B).

10.3.2.3. Changes in Selected Fecal Bacterial Populations Measured with qPCR

10.3.2.3.1. Shift in taxa relative to the core microbiota

Considering total *Eubacteria* (Table 4), compared with the abundances at the BL and apart from the blank control (BC) values, no sample showed statistically significant differences ($p > 0.05$), although the trend was characterized by a reduction for two of the three samples tested, in contrast to what was observed for lactose intolerant adults (Table 1).

Firmicutes e *Bacteroidetes* are the two principal bacterial phyla that live the adult human colon.

Considering *Firmicutes* (Tables 1), significant decreases at EP were observed for any sample ($P < 0.05$), with the highest reduction observed with SPF ($p < 0.05$).

Quantifications of the phylum *Bacteroidetes* (Table 1) showed changes especially at T1, when all samples except SPF showed significant differences compared to BL ($p < 0.05$). In particular, the fermentation of SPF+PRO was the best, significantly increasing the charge of this taxon at T1 to 1.49E+08 ± 3.92E+07 cells/mL ($p < 0.05$), i.e. 1.7 times more than the amount recorded by the unfermented control (SP).

Table 4. Quantification of Eubacteria, *Bacteroidetes*, and *Firmicutes*.

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Eubacteria	**BL**	**T1**	**EP**	
SP	2.68E+09 ± 1.52E+08	-0.14^{AB}	-0.22^{B}	*0.706600*
SPF	2.68E+09 ± 1.52E+08	0.51^{B}	0.06^{AB}	*0.167020*
SPF+pro	2.68E+09 ± 1.52E+08	-0.69^{A}	-0.01^{AB}	*0.101315*
BC	2.68E+09 ± 1.52E+08^{ab}	-0.23^{bAB}	0.48^{acA}	*0.023481*
		0.009723	*0.28006*	***p* value**
Firmicutes	**BL**	**T1**	**EP**	
SP	5.98E+08 ± 3.08E+07^{a}	-0.55^{bAB}	-0.35^{bA}	*< 0.000001*
SPF	5.98E+08 ± 3.08E+07^{a}	-0.49^{bA}	-1.04^{cC}	*< 0.000001*
SPF+pro	5.98E+08 ± 3.08E+07^{a}	-0.71^{bB}	-0.84^{cBC}	*< 0.000001*
BC	5.98E+08 ± 3.08E+07^{a}	-0.75^{bB}	-0.86^{bB}	*< 0.000001*
		0.000426	*<0.000001*	***p* value**

Bacteroidetes	**BL**	**T1**	**EP**	
SP	5.56E+07 ± 8.71E+06^{a}	0.83^{bB}	0.02^{aB}	*< 0.000001*
SPF	5.56E+07 ± 8.71E+06	1.08^{B}	0.70^{BC}	*0.199912*
SPF+pro	5.56E+07 ± 8.71E+06^{a}	1.39^{bB}	1.02^{cC}	*0.000347*
BC	5.56E+07 ± 8.71E+06^{a}	2.65^{bA}	2.36^{cA}	*< 0.000001*
		< 0.000001	*< 0.000001*	***p* value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SP = unfermented whey; SPF = fermented whey; SPF+PRO = fermented whey added with probiotics; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

10.3.2.3.2. Shift in commensal and beneficial taxa

Among the beneficial bacteria considered (Table 5), *Lactobacillales* and *Bifidobacteriaceae* behaved differently during fermentation.

Considering *Lactobacillales*, as observed for lactose intolerants (Table 2), a reduction in their abundance was also observed with healthy adults with all samples (Table 5). Interestingly, again, as with lactose intolerants, the least reduction was observed with SP.

In contrast, *Bifidobacteriaceae* increased with each substrate, but significantly only for SP ($p < 0.05$). Similarly, to the experiments conducted with lactose intolerant adults (Table 2), the *Clostridium IV* group was significantly reduced after any fermentation compared with BL ($p < 0.05$). In general, whey samples did not foster beneficial microbes, with the exception of the *Bifidobacteriaceae* family. This is a positive aspect since, through the production of SCFA, *Bifidobacteriaceae* are able to reduce pH, inhibiting the growth of pathogenic bacteria and promoting a probiotic effect on the gut induced by whey. In this regard, *Bifidobacteriaceae* not only decrease intestinal pH, but also enhance lysozyme activity and facilitate the destruction of certain pathogenic bacteria (Sanchez-Moya et al., 2017).

Table 5. Quantification of commensal beneficial taxa.

qPCR Target	**Cells/mL**	**Log$_2$(F/C)**		**MANOVA**
Lactobacillales	**BL**	**T1**	**EP**	
SP	1.56E+03 ± 4.04E+02^{a}	-0.13^{aA}	-0.99^{bA}	*< 0.000001*
SPF	1.56E+03 ± 4.04E+02^{a}	-1.69^{bBC}	-1.85^{bB}	*0.000027*
SPF+pro	1.56E+03 ± 4.04E+02^{a}	-0.63^{bB}	-1.88^{bB}	*0.004990*
BC	1.56E+03 ± 4.04E+02^{a}	-6.46^{bC}	-5.87^{cB}	*< 0.000001*
		< 0.000001	*0.000001*	***p* value**
Bifidobacteriaceae	**BL**	**T1**	**EP**	
SP	3.68E+07 ± 6.60E+06^{a}	-0.78^{bB}	0.32^{c}	*< 0.000001*
SPF	3.68E+07 ± 6.60E+06	0.31^{A}	0.04	*0.519841*
SPF+pro	3.68E+07 ± 6.60E+06	-0.04^{AB}	0.02	*0.991816*
BC	3.68E+07 ± 6.60E+06^{a}	0.35^{bA}	0.39^{b}	*0.000009*
		0.009116	*0.213059*	***p* value**

***Clostridium* Group IV**	**BL**	**T1**	**EP**	
SP	8.59E+07 ± 4.39E+07^{a}	-2.43^{bA}	-2.81^{c}	*< 0.000001*
SPF	8.59E+07 ± 4.39E+07^{a}	-4.82^{bB}	-3.59^{b}	*< 0.000001*
SPF+pro	8.59E+07 ± 4.39E+07^{a}	-2.79^{bA}	-1.89^{c}	*< 0.000001*
BC	8.59E+07 ± 4.39E+07^{a}	-3.70^{bB}	-1.97^{c}	*< 0.000001*
		0.000007	*0.331772*	***p* value**
B. longum	**BL**	**T1**	**EP**	
SP	1.05E+07 ± 1.95E+06^{a}	-0.26^{bB}	0.64^{c}	*< 0.000001*
SPF	1.05E+07 ± 1.95E+06	0.24^{A}	0.25	*0.552987*
SPF+pro	1.05E+07 ± 1.95E+06	-0.33^{AB}	0.15	*0.553496*
BC	1.05E+07 ± 1.95E+06^{a}	0.09^{bAB}	0.09^{b}	*0.001200*
		0.025621	*0.599964*	***p* value**

Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA *p* value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SP = unfermented whey; SPF = fermented whey; SPF+PRO = fermented whey added with probiotics; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

10.3.2.3.3. Shift in commensals opportunistic taxa

As observed with lactose intolerant adults, even with healthy adults any substrate tested was able to support the growth of *Enterobacteriaceae* from BL to EP, although SPF and SPF+PRO without significant differences ($p > 0.05$).The same trend was observed within this family, in fact, fermentations of all sera increased the total *E. coli*. For both taxa, the increase to EP was however smaller in SPF+PRO than in SPF.

In contrast to what was seen with lactose intolerants, with healthy adults any substrate reduced *Clostridium group I*, with SPF as the sample that reduced it the most.

Overall, our results did not show much reduction in the opportunistic bacterial taxa considered. This is due to the nature of the samples under study, as they are rich in proteins of animal origin. However, it is interesting to note the differences between the various samples.

Although from our results SP always limited the growth of the opportunists the most, if we only consider the fermented samples, the best results were obtained from SPF.

Table 6. Quantification of commensal opportunistic taxa

qPCR Target	**Cells/mL**	**Log_2(F/C)**		**MANOVA**
Enterobacteriaceae	**BL**	**T1**	**EP**	
SP	2.66E+07 ± 7.75E+06^{a}	1.04^{bB}	1.59^{cB}	*< 0.000001*
SPF	2.66E+07 ± 7.75E+06	1.64^{A}	2.55^{A}	*0.138294*
SPF+pro	2.66E+07 ± 7.75E+06	1.98^{B}	0.71^{AB}	*0.145309*
BC	2.66E+07 ± 7.75E+06^{a}	3.34^{bAB}	3.82^{cA}	*0.000003*

		0.017779	*0.008665*	***p* value**
***Clostridium* Group I**	**BL**	**T1**	**EP**	
SP	1.61E+07 ± 2.14E+07^{a}	-2.14^{b}	-0.04^{c}	*< 0.000001*
SPF	1.61E+07 ± 2.14E+07	-0.11	-0.73	*0.845120*
SPF+pro	1.61E+07 ± 2.14E+07	-0.91	-0.16	*0.272498*
BC	1.61E+07 ± 2.14E+07^{a}	-1.43^{b}	-0.04^{c}	*< 0.000001*
		0.253304	*0.055141*	***p* value**
Escherichia coli* (total)	**BL**	**T1**	**EP**	
SP	6.22E+05 ± 8.12E+04^{a}	-0.33^{bB}	0.73^{cA}	*< 0.000001*
SPF	6.22E+05 ± 8.12E+04	2.25^{A}	2.29^{AB}	*0.145196*
SPF+pro	6.22E+05 ± 8.12E+04	0.86^{B}	1.57^{A}	*0.491072*
BC	6.22E+05 ± 8.12E+04^{a}	0.40^{aB}	2.12^{bB}	*< 0.000001*
		0.005739	*0.000692*	***p* value**

*This taxon was amplified by targeting cell division protein (FtsZ) rDNA; Different capital letters indicate significance difference within a column; Different lower case letters indicate significance difference within a row according to MANOVA model followed by Tukey's HSD test ($p < 0.05$). MANOVA P value stands for italicized numbers relative to Time effect on rows and to Matrix effect on columns. SP = unfermented whey; SPF = fermented whey; SPF+PRO = fermented whey added with probiotics; BC = Blank Control; BL = Baseline; T1 = 18 h of fermentation; EP = 24 h of fermentation.

10.4. Conclusions

Total milk production is over 801 million tons, of which over 37% is processed into cheese or other coagulated products and 30% is used to produce butter. During the processing of these products, approximately 80-90% is whey, which is discharged as waste (Birania et al., 2021). To promote sustainable development, it is necessary to find sustainable ways of using whey, such as to produce beverages.

However, whey has a high lactose content, which can make this product unsuitable for consumers who are unable to digest lactose, about 70% worldwide.

To meet the needs of these consumers, many lactose-free dairy products are being developed, the consumption of which is also extended to healthy individuals when only one member in a family is lactose intolerant.

For this reason, the present study aimed to investigate the effect of fermented whey on the intestinal microbiota of both healthy subjects and lactose-intolerant individuals.

The results showed that fermented whey (SPF) has a positive effect on the gut flora of healthy subjects, increasing the production of beneficial metabolites (e.g. SCFA) and reducing the amount of potentially toxic metabolites (e.g. Phenol and p-Cresol). However, the results related to the microbiota showed a greater positive effect with SP, fostering the proliferation of beneficial microbial taxa, including *Bifidobacteriaceae*, and the reduction of negative microbial populations, including potentially toxic *E. coli*, although without significant differences compared to other samples.

In the case of lactose intolerant subjects, however, in terms of volatile analysis, the same positive result was obtained with unfermented whey (SP), but promising results were also obtained with fermented whey (SPF). The addition of probiotics to fermented whey was found to be more desirable for lactose intolerant people.

10.5. References

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10.6. Supplementary materials

Table S1. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	**Target**	**Sequenza 3'-5'**	**Bp ***	**Reference**
Eubacteria	V3-V4 16 S	Eub518-R: ATTACCGCGGCTGCTGG Eub338-R: ACTCCTACGGGAGGCAG	147	Lane *et al*, 1992
Bacteroidetes	V3-V4 16 S	Bact934F: GGARCATGTGGTTTAATT Bact1060R: AGCTGACGACAACCATG	250	Guo *et al*, 2008
Firmicutes	V3-V4 16 S	Firm934F: GGAGYATGTGGTTTAATT Eub338R: ACTCCTACGGGAGGCAG	300	Guo *et al*, 2008

Enterobacteriaceae	V3-V4 16 S	Enterobac-f: TGCCGTAACTTCGGGAG Enterobac-r: TCAAGGACCAGTGTTCAG	450	Bartosh *et al*, 2004
E. coli (total)	FtsZ	EcFtsZ-F: GGTATCCTGACCGTTGCT EcFtsZ-R: ATACCTCGGCCCAGAACT	250	Zhou *et al*, 1994
Lactobacillales	V3-V4 16 S	F-Lac: GCAGCAGTAGGGAATCT R-Lac: GCATTYCACCGCTACACA	340	Walter *et al*, 2001
Bifidobacteriaceae	RecA	RecAf: CGTYTCBCAGCCGGAYA RecAr: CCARVGCRCCGGTCATC	220	Masco et al., 2006
B. longum	V3-V4 16 S	Blon-f: GATTCTGGCTCAGGATGA Blon-r: CTGATAGGACGCGACCC	220	Chen *et al*, 2007
Clostridium group I	V3-V4 16 S	ClosI-F: TACCHRAGGAGGAAGCCAC ClosI-R: GTTCTTCCTAATCTCTACGCAT	148	Bartosh *et al*, 2004
Clostridium group IV	V3-V4 16 S	ClosIV-f: TTAACACAATAAGTWATC ClosIV-r: ACCTTCCTCCGTTTTGTC	400	Goldberg *et al*, 2013

*Bp: base pair

Table S2. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors significant among the food matrices.

VOCs	% of contribution of VOCs descriptors					MANOVA
	Baseline	**SP**	**SPF**	**SPF+pro**	**BC**	***P value***
Acetic acid	6.35^{bc}	34.42^{a}	28.26^{ab}	19.12^{ab}	11.84^{bc}	0.015167
Propanoic acid	3.50^{b}	51.28^{a}	15.82^{b}	19.45^{b}	9.95^{b}	0.048358
Pentanoic acid, 3-methyl-	0.65^{b}	77.05^{a}	0.00^{b}	22.30^{a}	0.00^{b}	0.013195
Pentanoic acid	6.44^{b}	34.13^{a}	20.94^{ab}	34.73^{a}	3.76^{b}	0.003024
Octanoic acid	23.42^{b}	13.99^{b}	5.88^{b}	6.19^{b}	50.53^{a}	0.000474
Ethyl alcohol	0.51^{b}	39.78^{a}	33.66^{ab}	17.05^{ab}	8.99^{b}	0.004580
1-Propanol	2.26^{b}	45.45^{a}	28.29^{a}	12.06^{b}	11.94^{b}	0.000926
1-Butanol	18.77^{ab}	29.13^{a}	15.61^{ab}	31.64^{ab}	4.84^{b}	0.007576
2-Hexanol	30.53^{ab}	30.94^{ab}	23.21^{ab}	15.32^{bc}	0.00^{c}	0.004048
3-Heptanol	26.36^{b}	41.35^{a}	23.12^{b}	9.17^{c}	0.00^{d}	< 0.000001
1-Pentanol	45.27^{a}	20.44^{b}	10.28^{b}	24.01^{b}	0.00^{c}	0.000006
1-Hexanol	29.27^{ac}	28.86^{c}	14.08^{a}	27.78^{ac}	0.00^{b}	0.000047
1-Hexanol, 2-ethyl-	25.69^{a}	18.59^{ab}	22.27^{a}	21.83^{a}	11.63^{b}	0.002296
1-Nonanol	14.47^{bc}	38.18^{ab}	16.49^{ac}	30.87^{a}	0.00^{c}	0.000128
Phenylethyl Alcohol	0.00^{b}	18.89^{ab}	16.63^{ab}	16.97^{ab}	47.51^{a}	0.009762
Phenol	5.47^{b}	9.39^{b}	6.18^{b}	9.40^{b}	69.56^{a}	0.014901
Phenol, 2,4-bis(1,1-dimethylethyl)-	12.47^{b}	11.78^{b}	11.57^{b}	13.44^{b}	50.74^{a}	0.002497
Indole	0.46^{b}	0.19^{b}	0.22^{b}	0.20^{b}	98.92^{a}	< 0.000001

1H-Indole, 3-methyl-	21.06^{ab}	11.09^{b}	12.13^{b}	11.10^{b}	44.62^{a}	0.000611
Butanal	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.000000
Pentanal	36.48^{a}	10.91^{ab}	30.75^{a}	21.85^{ab}	0.00^{b}	0.007842
Heptanal	29.14^{a}	8.92^{ab}	19.33^{b}	19.97^{ab}	22.63^{ab}	0.040288
2-Nonenal, (E)-	11.04^{ab}	24.80^{ab}	30.58^{a}	25.03^{ab}	8.54^{b}	0.012346
Benzaldehyde	52.95^{a}	9.51^{ab}	7.81^{b}	4.94^{b}	24.79^{c}	0.000004
3-Thiophenecarboxaldehyde	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.000001
Benzaldehyde, 2,4-dimethyl-	34.70^{ab}	8.69^{b}	3.50^{b}	3.21^{b}	49.90^{a}	0.000198
Acetone	47.21^{a}	19.30^{ab}	0.00^{b}	29.05^{ab}	4.44^{ab}	0.016261
2-Butanone	2.58^{b}	1.08^{b}	0.96^{b}	1.54^{b}	93.85^{a}	0.000002
2,3-Butanedione	37.60^{a}	13.35^{a}	29.63^{ab}	19.42^{a}	0.00^{b}	0.001603
2-Hexanone	25.76^{a}	25.33^{a}	27.02^{a}	21.89^{a}	0.00^{b}	0.000484
2,3-Pentanedione	11.54^{abc}	15.59^{ab}	41.93^{ac}	30.93^{c}	0.00^{b}	0.006443
3-Hexanone, 5-methyl-	28.37^{a}	15.46^{a}	28.08^{a}	28.09^{a}	0.00^{b}	0.000335
2-Hexanone, 4-methyl-	26.20^{a}	25.39^{a}	26.02^{a}	22.39^{a}	0.00^{b}	0.000041
3-Hexen-2-one	38.98^{a}	14.91^{a}	23.82^{a}	22.29^{a}	0.00^{b}	0.000065

^{abc} Different letters indicate statistical significance by Tuckey's HSD test ($p < 0.05$).

Table S3. MANOVA categorical descriptors for the volatilome, categorized for the time of fermentation. % of contribution of VOCs descriptors significant among the time.

VOCs	% of contribution of VOCs descriptors MANOVA			
	Baseline	**16h**	**24h**	***P value***
Ethyl alcohol	1.01^{b}	34.18^{ab}	64.81^{a}	0.028305
1-Pentanol	65.68^{a}	10.94^{b}	23.38^{b}	0.009636
Butanal	100.00^{a}	0.00^{b}	0.00^{b}	< 0.000001
Benzaldehyde	69.24^{a}	15.17^{b}	15.59^{b}	0.000210
3-Thiophenecarboxaldehyde	100.00^{a}	0.00^{b}	0.00^{b}	< 0.000001
Acetone	66.87^{a}	0.00^{b}	33.13^{a}	0.005348
3-Hexen-2-one	56.18^{a}	21.96^{b}	21.85^{b}	0.044586

^{abc} Different letters indicate statistical significance by Tuckey's HSD test ($p < 0.05$).

Table S4. Baseline values of beneficial VOCs in mM.

VOCs	**Baseline (mM ± SD)**		
Acetic acid	16.29	±	2.20
Butanoic acid	18.19	±	2.46
Propanoic acid	1.87	±	0.33

Table S5. Baseline values of detrimental VOCs in mM.

VOCs	**Baseline (mM ± SD)**

Phenol	5.98	±	1.23
Phenol, 4-methyl-	132.99	±	54.01
Indole	43.49	±	4.10
Skatole	27.71	±	2.14

Table S6. MANOVA categorical descriptors for the volatilome, categorized for the type of matrix. % of contribution of VOCs descriptors significant among the food matrices.

VOCs	% of contribution of VOCs descriptors					MANOVA
	Baseline	**SP**	**SPF**	**SPF+pro**	**BC**	***P value***
Acetic acid	0.00^{d}	27.50^{b}	44.38^{a}	12.53^{cd}	15.59^{c}	< 0.000001
Benzoic acid, methyl ester	40.56^{a}	7.02^{bc}	33.28^{b}	19.14^{abc}	0.00^{c}	0.002063
Butanoic acid	1.73^{c}	24.62^{ab}	32.63^{a}	15.23^{bc}	25.79^{ac}	0.000432
Hexanoic acid	1.35^{c}	27.56^{b}	51.74^{a}	8.54^{bc}	10.81^{bc}	0.000060
Pentanoic acid	1.06^{c}	26.58^{bc}	47.80^{a}	9.25^{c}	15.31^{bc}	0.000001
Pentanoic acid, 3-methyl-	0.29^{c}	20.31^{b}	52.64^{a}	10.09^{bc}	16.68^{b}	< 0.000001
Propanoic acid	2.20^{c}	22.67^{b}	55.41^{a}	7.58^{b}	12.13^{b}	0.000001
Propanoic acid, 2,2-dimethyl-	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.000006
1-Heptanol	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.000001
1-Hexanol	21.23^{ab}	25.08^{ab}	15.33^{a}	4.14^{b}	34.21^{a}	0.000057
1-Hexanol, 2-ethyl-	12.92^{ac}	21.28^{c}	10.26^{c}	21.89^{b}	33.65^{a}	0.000065
1-Octanol	26.44^{ab}	30.44^{a}	15.26^{c}	12.35^{bc}	15.51^{bc}	0.000376
1-Pentanol	1.96^{b}	18.32^{ab}	16.26^{ab}	26.98^{a}	36.48^{ab}	0.010710
1-Propanol	9.03^{c}	27.01^{ab}	30.36^{ab}	27.42^{c}	6.18^{ab}	0.000082
5-Hexen-2-ol, (.+/-.)-	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.000001
Benzyl Alcohol	0.00^{c}	26.27^{b}	0.00^{c}	40.33^{a}	33.40^{a}	< 0.000001
Cyclohexanol, 3,3,5-trimethyl-, acetate, cis-	95.30^{a}	1.39^{b}	0.00^{b}	0.53^{b}	2.78^{b}	< 0.000001
Phenol	0.31^{b}	51.62^{a}	2.09^{c}	37.17^{b}	8.81^{b}	< 0.000001
Phenylethyl Alcohol	4.36^{b}	37.50^{a}	10.30^{b}	31.92^{a}	15.91^{b}	0.002142
Indole	1.08^{b}	31.46^{a}	15.58^{ab}	25.08^{ab}	26.79^{b}	0.000764
2-Butenal, 2-methyl-	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.000001
2-Nonenal, (E)-	54.97^{a}	0.00^{b}	0.00^{b}	45.03^{a}	0.00^{b}	< 0.000001
Benzaldehyde	11.02^{b}	20.38^{b}	34.97^{a}	20.02^{b}	13.61^{b}	0.000071
Benzaldehyde, 3,5-ethyl-	18.64^{abc}	29.03^{ab}	21.01^{abc}	16.31^{bc}	15.02^{bc}	0.002800
Butanal	26.40^{abc}	8.92^{d}	19.17^{abcd}	16.71^{abcd}	28.81^{abc}	0.005537
Hexanal	36.81^{a}	14.12^{c}	20.21^{bc}	28.86^{ab}	0.00^{d}	0.000001
Octanal	90.59^{a}	0.18^{b}	8.51^{b}	0.72^{b}	0.00^{b}	< 0.000001
Propanal, 3-(methylthio)-	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.000001
2,3-Butanedione	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.000001
2-Butanone	15.28^{ab}	16.58^{a}	19.00^{ab}	28.55^{b}	20.58^{ab}	0.028652
2-Butanone, 4-hydroxy-	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.000007
2-Hexanone, 4-methyl-	28.66^{abc}	11.41^{c}	13.90^{c}	17.48^{abc}	28.55^{ab}	0.006412
3(2H)-Furanone, dihydro-2-methyl-	100.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	< 0.000001
3-Hexanone, 5-methyl-	66.44^{a}	24.90^{b}	8.66^{bc}	0.00^{c}	0.00^{c}	0.000031

3-Penten-2-one, 4-methyl-	31.85[a]	10.36[b]	12.20[ab]	25.06[ab]	20.53[ab]	0.016615
4-Heptanone, 2,6-dimethyl-	22.96[ab]	17.34[b]	17.31[b]	18.33[b]	24.06[a]	0.005494
4-Isopropyl-1,3-cyclohexanedione	26.91[a]	31.98[a]	20.43[a]	20.67[a]	0.00[b]	0.001931
Acetone	21.28[b]	16.19[b]	17.26[b]	29.22[a]	16.05[b]	0.012884
Acetophenone	43.75[a]	10.17[b]	17.73[ab]	28.35[a]	0.00[b]	0.001513
Dihydro-2-(3H)-thiophenone	0.00[b]	8.32[b]	20.93[b]	63.76[a]	6.99[b]	0.000026
Mercaptoacetone	0.00[b]	0.00[b]	0.00[b]	100.00[a]	0.00[b]	0.000014
2-Hexanone	38.14[a]	16.68[b]	12.94[b]	13.72[b]	18.52[b]	0.000068

[abc] Different letters indicate statistical significance by Tuckey's HSD test ($p < 0.05$).

Table S7. MANOVA categorical descriptors for the volatilome, categorized for the time of fermentation. % of contribution of VOCs descriptors significant among the food matrices.

VOCs	% of contribution of VOCs descriptors MANOVA			
MANOVA	**BL**	**16h**	**24h**	***p value***
Butanoic acid	3.39[b]	47.57[a]	49.03[a]	0.009328
Propanoic acid, 2,2-dimethyl-	100.00[a]	0.00[b]	0.00[b]	0.000002
1-Heptanol	100.00[a]	0.00[b]	0.00[b]	< 0.000001
5-Hexen-2-ol, (.+/-.)-	100.00[a]	0.00[b]	0.00[b]	< 0.000001
Cyclohexanol, 3,3,5-trimethyl-, acetate, cis-	98.00[a]	0.60[b]	1.41[b]	< 0.000001
Indole	2.19[a]	58.26[a]	39.55[b]	< 0.000001
2-Butenal, 2-methyl-	100.00[a]	0.00[b]	0.00[b]	< 0.000001
2-Nonenal, (E)-	90.02[a]	9.98[b]	0.00[b]	0.029975
Octanal	98.08[a]	0.10[b]	1.82[b]	< 0.000001
Propanal, 3-(methylthio)-	100.00[a]	0.00[b]	0.00[b]	< 0.000001
2,3-Butanedione	100.00[a]	0.00[b]	0.00[b]	< 0.000001
2-Butanone, 4-hydroxy-	100.00[a]	0.00[b]	0.00[b]	< 0.000001
3(2H)-Furanone, dihydro-2-methyl-	100.00[a]	0.00[b]	0.00[b]	< 0.000001
3-Hexanone, 5-methyl-	88.74[a]	11.26[b]	0.00[b]	0.000747
3-Penten-2-one, 4-methyl-	47.71[a]	21.16[b]	31.13[a]	0.044801
4-Heptanone, 2,6-dimethyl-	37.02[a]	28.00[b]	34.99[a]	0.015245
Acetophenone	65.21[a]	24.29[b]	10.50[b]	0.045323
2-Hexanone	55.01[a]	20.98[c]	24.01[c]	0.000014

[abc] Different letters indicate statistical significance by Tuckey's HSD test ($p < 0.05$).

Table S4. Baseline values of beneficial VOCs in mM.

VOCs	**Baseline (mM)**		
Acetic acid	n.d.		
Butanoic acid	13.17	±	2.44
Propanoic acid	2.41	±	1.11

CASE STUDY 7: Lactose-free whey

Table S5. Baseline values of detrimental VOCs in mM.

VOCs	**Baseline (mM)**		
Phenol	14.37	±	4.10
Phenol, 4-methyl-	147.13	±	50.79
Indole	190.03	±	77.27

11. Engineered strain of *Lactococcus lactis* as a biotechnological approach to reduce lactose intolerance

This research activity was carried out from 29 March 2022 to 30 June 2022 at the IATA-CSIC in Valencia (Spain) with the supervision of Dr. Maria J. Yebra. A research article recipient of these results is in preparation under invitation for submission on *Microorganisms*

11.1. Introduction

Lactase deficiency is a widespread condition among adults, a consequence of the non-persistence of lactase due to a progressive decline in activity (Leis et al., 2020). In subjects with lactase deficiency, probiotics reduce the symptoms of swelling thanks to the presence of microbial lactase. Generally, lactase for industrial purposes is obtained from yeasts and fungi, such as *Aspergillus oryzae*, *Kluyveromyces fragilis* and *Kluyveromyces lactis*. However, although the enzymes purified from Aspergillus are still widely used, the extraction method is expensive. The GH2 beta-galactosidases of lactic acid bacteria (LAB) are pre-dominantly of the heterodimeric type LacLM, encoded by the two overlapping genes lacL and lacM, however there are also GH2 di- or oligomeric beta-galactosidases of the LacZ type, encoded by the single lacZ gene. In addition to hydrolyzing lactose, β- galactosidases can also catalyze transgalactosylation reactions, transferring portions of galactosyl to e.g. lactose to a suitable acceptor molecule. When lactose is the primary acceptor, galacto-oligosaccharides (GOS) are obtained. Nguyen et al. (2015) screened several *Lactobacillus* isolates and found that one strain of *L. reuteri* exhibited high β-galactosidase activity with significant transferase activity. The activity levels obtained with the wild strain, however, were too low to be attractive from an application point of view. To enhance these low yields, the coding regions of the two overlapping genes lacL and lacM (lacLM) were cloned and overexpressed in a standard expression host, *Escherichia coli*. Heterologous expression in *E. coli* resulted in effective overexpression of be-ta-galactosidase however *E. coli* is not a suitable host for food-bound enzymes; therefore, overproduction of this enzyme has been reported in *L. plantarum* (Nguyen et al., 2015). In the literature there are also studies in which, with a chemical mutagenesis protocol, the production of lactase was successfully increased by *Bifidobacterium breve* and *B. bifidum* (Ibrahim and O'Sullivan, 2000) and by *L. reuteri* (Alezzeh et al., 2011). The use of LAB in which an overexpression of the

lactase enzyme has been obtained would allow to overcome the high cost of extracting the enzyme, as it would be carried directly by the microorganism. The engineered strain could be useful for industrial delactosation processes of dairy products. This study aims to develop engineered LAB strains in which an overexpression of the lactase enzyme has been obtained.

11.2. Materials and methods

11.2.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. The Lactococcus lactis strains were grown at 30°C under static conditions in M17 medium (Oxoid, Thermo Fisher Scientific, USA) supplemented with 0.5% of glucose (M17G). The corresponding solid media were prepared by adding 1.5% agar. L. lactis transformants were selected on M17G with erythromycin (5 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg ml^{-1}).
Vector pT1NX (Schotte et al., 2000) was used for cloning and expression of bdg2A in *L. lactis*. *L. lactis* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, USA) as described by Holo and Nes (1989).

Table 1. Strains and plasmids used in this study

Lactococcus lactis	**Genotype or properties**	**Source**
MG1363	Plasmid and phage-free derivative of NCDO712	Gasson 1983
362	MG1363 containing pT1NX Erm^R	Schotte et al., 2000
558	MG1363 containing pT1-uspS-bdg2A-spaX; Erm^R	This work
559	MG1363 containing pT1-uspS-bdg2A; Erm^R	This work
Plasmids		
pT1NX	Contains the P1 constitutive promoter, usp45 secretion signal and spaX cell wall anchor Erm^R	Schotte et al., 2000
pT1-uspS-bdg2A-spaX	pT1NX containing uspS, bdg2A - encoding region and spaX	This work
pT1-uspS-bdg2A	pT1NX containing uspS and bdg2A - encoding region	This work

Erm^R, erythromycin resistance.

11.2.2. *DNA manipulation and sequencing*

Total DNA was isolated from *B. dentium* Y510 using the MasterPure DNA extraction Kit (Epicentre) following the manufacturer's protocols with some modifications (Rubio-del-Campo et al., 2020). Recombinant DNA techniques were performed according to the laboratory manuals (Sambrook et al. 1989). All PCRs were performed with the Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). DNA sequencing was performed by Eurofins Genomics (http://www.eurofinsgenomics.com) to confirm the correct sequence of the inserts. Sequence analyses were carried out with DNAMAN 4.0 for Windows (Lynnon BioSoft, Quebec, Canada).

11.2.3. Constructions of plasmids and strains

The coding region of bdg2A (Moya-Gonzalvez et al. 2021) was amplified by PCR using *B. dentium* Y510 chromosomal DNA as a template. The bdg2A gene was obtained with the primers Bdg2AXmaI (5'-TTTTCCCGGGATGTCGCATATCTTTTCCTC) and Bdg2ABglII (5'-TTTTAGATCTGTGAACAGCTCCAGCATCAC). The amplified DNA fragment was digested with XmaI and BglII and cloned into the replicative vector pT1NX (Schotte et al., 2000), which has been previously digested with NgoMIV and BamHI (these restriction enzymes have compatible cohesive ends with XmaI and BglII, respectively). The competent *L. lactis* MG1363 strain was electroporated with the ligation mixture and the transformants carrying pT1NX with bdg2A gene were screened for the presence of blue colonies. In the resulting plasmid, pT1-uspS-bdg2A-spaX, the bdg2A gene is inserted between the lactococcal usp45 secretion signal sequence and the sequence encoding the cell wall anchor of *Staphylococcus aureus* protein A (spaX). The bdg2A gene was also obtained with the primers Bdg2AXmaI and Bdg2ASpeI (5'-TTTTACTAGTTCAGAACAGCTCCAGCATC). The amplified DNA fragment was digested with XmaI and SpeI and cloned into pT1NX digested with NgoMIV and SpeI. Blue colonies were selected as described above and in the resulting plasmid, pT1-uspS-bdg2A, the bdg2A gene is inserted after the lactococcal usp45 secretion signal sequence. In both plasmids, pT1-uspS-bdg2A-spaX and pT1-uspS-bdg2A, the bdg2A gene was expressed under the control of the lactococcal P1 constitutive promoter. Transformants were selected on M17G plates containing erythromycin and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). One transformant containing each plasmid was selected, and they were named 558 (MG1363 [pT1-uspS-bdg2A-spaX]) and 559 (MG1363 [pT1- uspS-bdg2A]).

11.2.4. Culture of Lactococcus lactis recombinant strains with lactose and glucose

The *L. lactis* strains were grown overnight at 30°C under static conditions on M17G medium. These cultures were diluted to an OD_{550} of 0.1 in 100 µl of M17 medium supplemented with 20 mM of lactose or glucose. Bacterial growth was monitored during 28 h by spectrophotometric measurements every 30 min at 550 nm in 96-well plates at 30°C without shaking in a POLARstar Omega microplate reader (BMG Labtech, Offenburg, Germany). At least three independent biological replicates for each growth curve were obtained. Results were expressed as means ± standard deviations.

To determine the residual carbohydrates present in the supernatants from the *L. lactis* cultures, the cells were removed by centrifugation and the supernatants were filtrated and analyzed by high-performance liquid chromatography (HPLC) using an ICS3000 chromatographic system (Dionex) and a CarboPac PA100 column with pulsed amperometric detection. A gradient of NaOH (10 mM NaOH for 1min, 10–100 mM NaOH for 15 min and 100 mM for 2 min) was used at 27 °C and at a flow rate of 1 ml/min for the analysis of lactose, glucose, and galactose. These carbohydrates were confirmed by comparison of their retention times with those of standards.

11.2.5.β-Galactosidase activity

The *L. lactis strains* 362, 558, and 559 were cultured overnight at 30 °C on 50 ml of M17G. Cells were collected by centrifugation, washed with Tris–HCl buffer 50 mM, pH 7.5 and suspended in this buffer to an OD_{550} of 2. Cell-free crude extract was prepared as previously described (Garcia-Mantrana et al., 2016). The β-galactosidase enzyme activity was determined by measuring the 2-nitrophenol released (absorbance at 404 nm) from 2-nitrophenyl (NP)-β- D-galactopyranoside (oNPGal) at 37 °C in 96-well plates (POLARstar Omega microplate reader, BMG Labtech). The reaction mixtures (200 µl) containing 100 mM Tris–HCl buffer pH 7.0, 5 mM oNPGal were started by adding 80 µl of culture supernatant, 10 µl of whole cells, or 40 µl of cell-free crude extract.

11.2.6. Determination of plasmid stability

The stability of the plasmids constructed in this study was tested under non-selective conditions. The 362, 558, and 559 strains were inoculated in 10 mL of M17 with glucose or lactose as carbon source and grown for 24 h. The culture was diluted and inoculated in a new medium and cultured under the same conditions. Up to 100 generations, the plasmid stability was assessed by plating diluted aliquots on M17G agar medium with X-gal and comparing the white colony counts in respect to the blue colony counts.

11.3. Results and discussion

11.3.1. Cell wall attached and extracellular expression of β-Galactosidase bdg2A in Lactococcus lactis

The higher β-Galactosidase activity (**Figure 1**) with whole cells was detected in strain 558, the one with the enzyme attached to the cell wall. The 559 strain showed a similar activity in the analyses with whole cells, suggesting that a high proportion of the enzyme is also retained at the cell surface. The 558 strain was also the strain with the higher activity in the crude extract, while as we expected the higher activity in the supernatant resulted from strain 559, showing that this strain can efficiently secrete the β-galactosidase bdg2A in an active form.

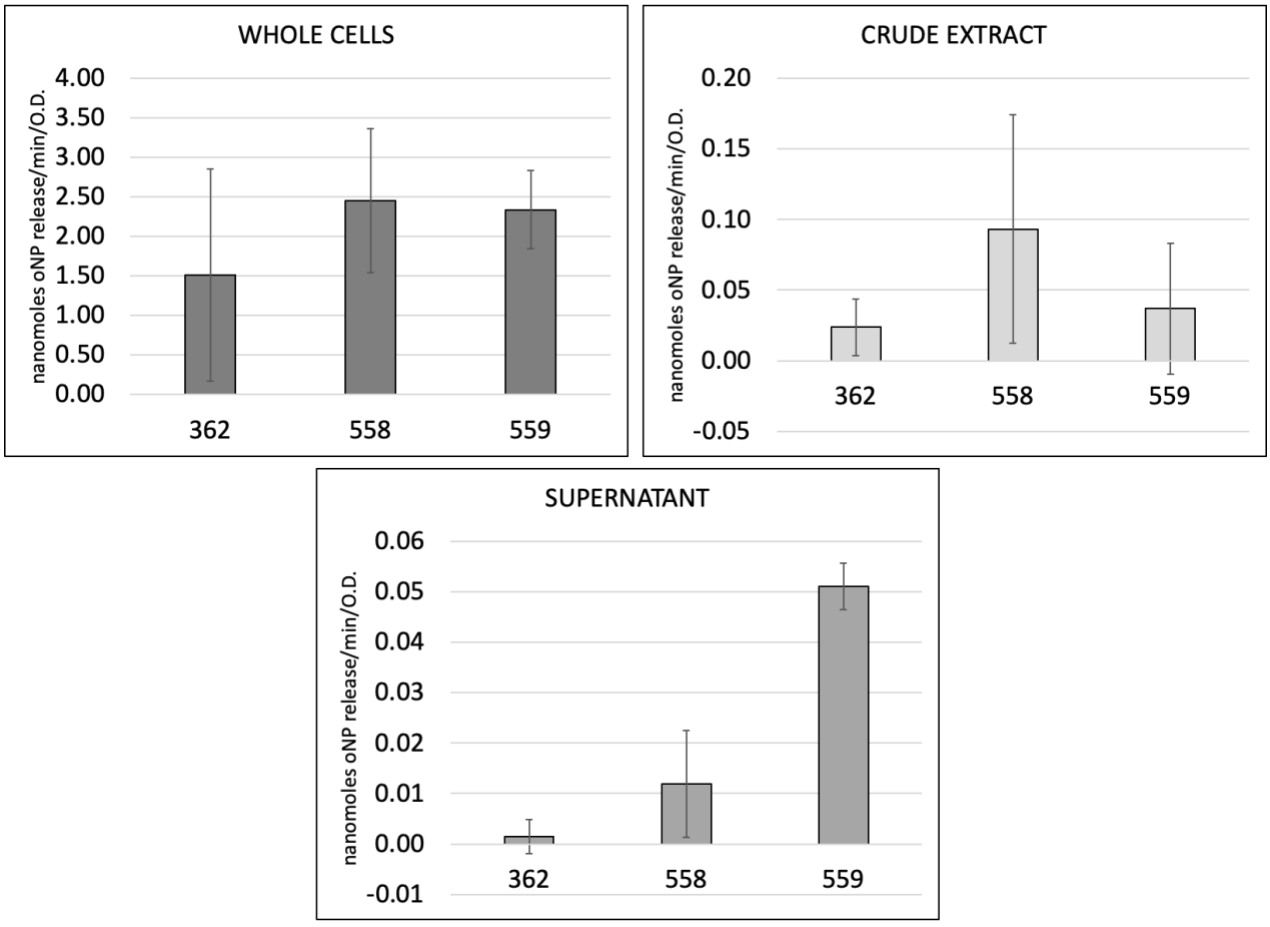

Figure 1. β-Galactosidase activity in whole cells, crude extracts, and supernatant of *Lactococcus lactis* strains 362 (pT1NX), 558 (pT1uspSbdg2AspaX) and 559 (pT1uspSbdg2A). The activity is expressed as nanomoles oNP release/min/O.D.

11.3.2. Recombinant Lactococcus lactis strains producing β-Galactosidase Bdg2A are able to ferment lactose

In contrast to the control strain 362, both recombinant strains 558 and 559 were able to growth on lactose, demonstrating the functionality of the expressed β-galactosidase Bdg2A in these strains (**Figure 2**). The results showed that strain 559, compared with control and 558 strains, reached a lower final OD on glucose. However, on lactose, strain 559 showed the most efficient growth, reaching values comparable to that with glucose though the growth started later. The growth of strain 558 with lactose was less efficient than that with glucose. Growth curves of the control strain 362 confirmed that this strain was unable to use lactose.

To confirm that lactose was fermented by the *L. lactis* strains 558 and 559, the supernatants of the cultures were analyzed for sugar content by high-performance liquid chromatography (Dionex system). Lactose and their constituent monosaccharides, galactose and glucose, were not found in the culture supernatants, indicating that they were metabolized (**Figure 3**).

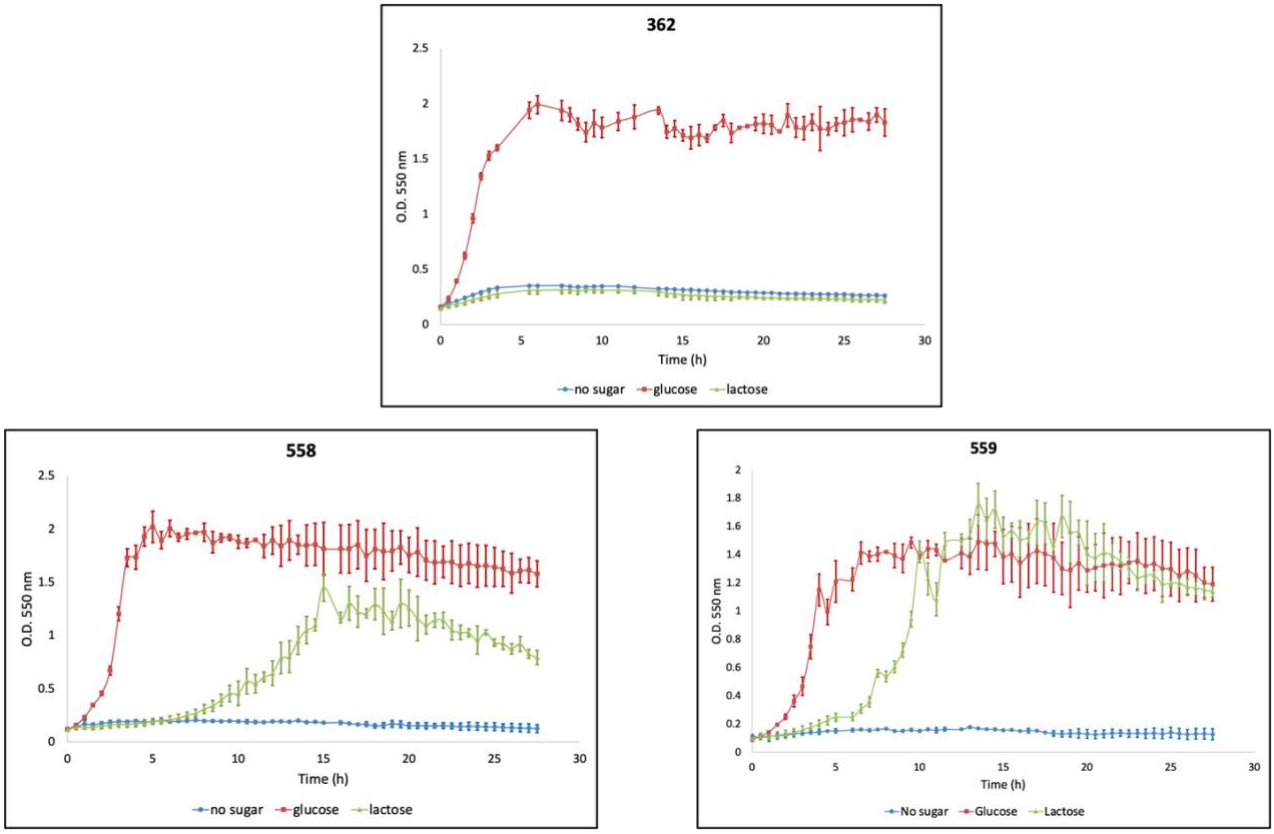

Figure 2. Growth curves of *Lactococcus lactis* strains 362 (pT1NX), 558 (pT1uspSbdg2AspaX) and 559 (pT1uspSbdg2A) with glucose or lactose as carbon source.

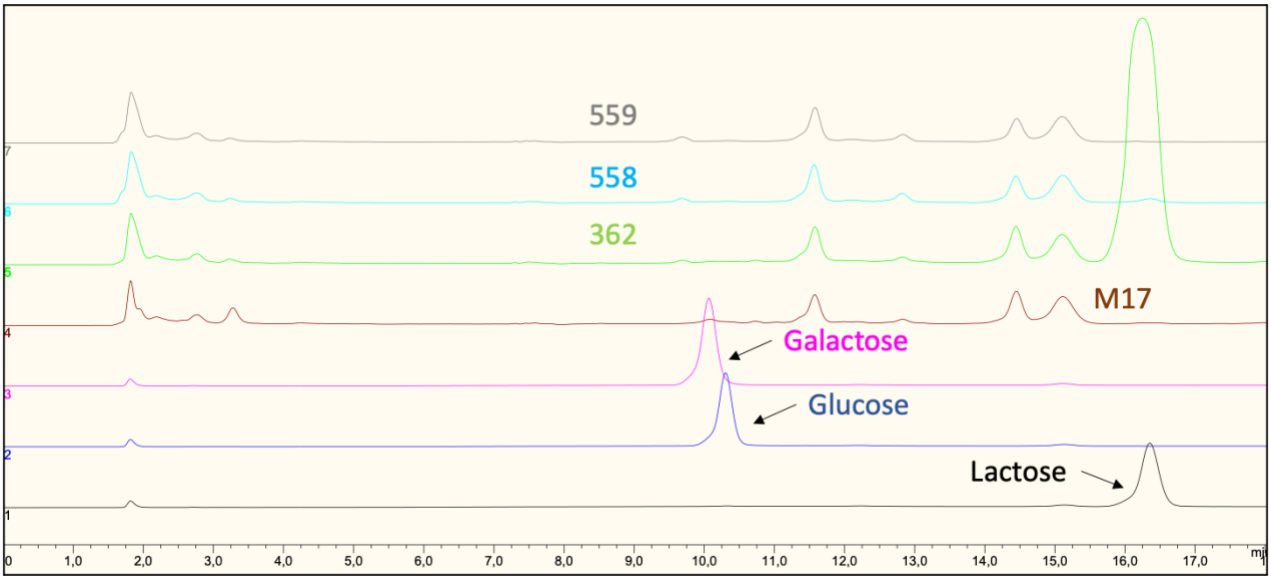

Figure 3. HPLC chromatograms (Dionex system) of the culture supernatants of *L. lactis* 362 (pT1NX), 558 (pT1uspSbdg2AspaX) and 559 (pT1uspSbdg2A) grown on M17 with lactose as carbon source, and of the standard compounds galactose, glucose, and lactose.

11.3.2. Stability of the plasmid

In both strains, 558 and 559, the stability of the plasmid was lower when cells were cultivated with lactose (**Figure 4**). The more unstable was 558. In fact, all the cells lost the plasmid after 40 generations. On the contrary, the 558 was the more stable when cultivated with glucose, although both 558 and 559 lost the plasmid after 96 generations.

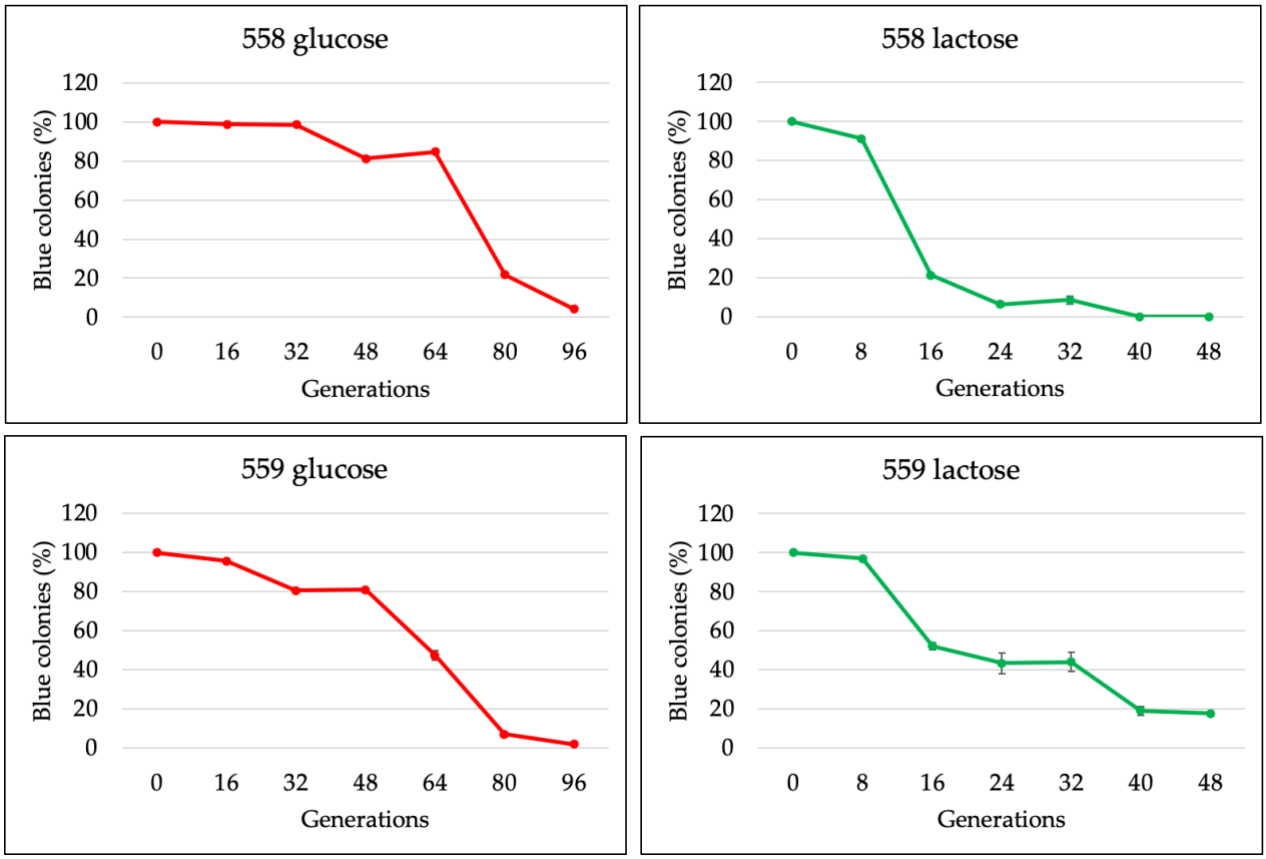

Figure 4. Plasmid stability in M17 with glucose or lactose as carbon source.

11.4. Conclusions

In this study, two recombinant strains of *L. lactis* expressing a *Bifidobacterium* β-galactosidase attached to the cell wall (strain 558) or released to the supernatant (strain 559) were constructed, Overall, the results showed that the best performant strain was 559. Its greater efficiency may be due to the significant enzymatic activity observed not only with the supernatant but also with whole cells, as confirmed by HPLC analyses. The latter, in fact, showed that 559 removed the greatest amount of lactose both using the cells and using the supernatant. In conclusion, 559 was a promising candidate for industrial application, also thanks to the greater stability of the plasmid when cells are grown in the presence of lactose as a carbon source.

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12. CASE STUDY 8: Maternal amoxicillin treatment perturbates piglets' colon microbiota

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12.1. Introduction

In the last decades, the swine has been acknowledged as one of the most important preclinical species for a wide variety of physiological patterns. Indeed, the swine species show close similarities with humans, and the employment of pigs in research trials seems to be more widely accepted by society in terms of ethical values (Ventrella et al., 2021).

One of the latest interesting applications of this model is the study of transport of endogenous and exogenous molecules, such as pharmacological compounds, during the lactation stage which is possible via passive or active transport mechanisms, since the endothelium does not constitute a major barrier to solute movement between blood and the interstitium (Shennan and Peaker, 2000). Transcellular transport requires solutes to cross the epithelial cell membranes, whereas paracellular transfer occurs between cells via leaky tight junctions (Nauwelaerts et al., 2021; Shennan and Peaker, 2000). In particular, pharmacological compounds can reside in one (or more) milk fractions such as casein, fat globules, or free in the aqueous acid whey; it was acknowledged that hydrophilic drugs accumulate in the liquid medium (Ozdemir et al., 2018).

Since 2019, the European project entitled ConcePTION aims at generating accurate knowledge about the use of medication during pregnancy and breastfeeding ("ConcePTION," n.d.) by means of different approaches. Out of the latter, in vitro, in vivo and in silico porcine trials have been established to generate data comparable and, most importantly translatable, to humans (Ventrella et al., 2019). Within said project, amoxicillin was chosen as the first test molecule since it is widely

used for therapeutic purposes in both human and porcine medicine, with well defined pharmacokinetics/pharmacodynamics (PK/PD) background data (Burch and Sperling, 2018).

Amoxicillin is a bactericidal antibiotic in the group of aminopenicillins. When given orally to juvenile, but yet not suckling pigs, the bioavailability of amoxicillin varies between 25 and 31%, and thus, substantial drug quantities may have a direct impact on the gut microbiota (Burch and Sperling, 2018). Indeed, swine gastrointestinal tract hosts a complex community of microorganisms, which compose the microbiota and take active part in immunity, digestive physiology and nutrients metabolism (Luo et al., 2022). The microbiota of newborns is mainly transferred from the sow at birth and then later from the sow's colostrum and milk, but it is also shaped by the surrounding environment (Isaacson and Kim, 2012; Luo et al., 2022). The microbiota of piglets is dominated by *Firmicutes*, and in particular by the orders *Lactobacillales* (Petri et al., 2010) and *Clostridiales* (Yang et al., 2021). The piglet's colon microbiota is inherited from the sow, not solely through milk, and among *Lactobacillales*, *Lactobacillaceae* early establish an important symbiosis that sculpture the intestinal epithelium up to the adult phase and bestow to the most beneficial effects derived from the microbiota (Petri et al., 2010).

In such regard, this study aims to evaluate the effect of sows' milk with different concentrations of amoxicillin, widely used as antibiotic in piggeries, on the perturbations of the newborns gut microbiota using an innovative *in vitro* colon model. We used MICODE (Nissen et al., 2021a, 2021b) modified by using piglets' feces from four healthy animals for a short-term colonic fermentation protocol (24 h) of different sow's milk containing different concentrations of amoxicillin residues in comparison to a sow's milk with no antibiotic and to a blank control. This system permitted to resemble *in vitro* the *in vivo* conditions of piglets' gut ecology, in line with the international call to reduce animal testing (Directive 2010/63/EU; Regulation (EU) 2019/1010). In particular, it serves to highlight the shift that happens in the core microbiota and in the related volatilome after colonic fermentation. The results were obtained coupling microbiomics (qPCR and Illumina sequencing) and metabolomics (SPME GC-MS) and studying several ecological indicators either related to microbes and molecules, as: i) microbial biodiversity, ii) microbial eubiosis, iii) shifts in the core microbiota at high or low taxonomical levels of selected opportunistic and beneficial commensals taxa, iv) production of postbiotics, v) production of detrimental compounds.

12.2. Materials and methods

12.2.1. Preparative

Conventional pregnant sows were purchased from a local farm, SUIMAX di Massimo Ferri (Via San Michele 718, Valsamoggia 40056 BO, Italy), chosen of the basis of the microbiological status of the facility and for the reproductive track records. All piglets included in the study were born from the above-mentioned sows in the experimental facility of the ANFI-ASA Unit, Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna (via Tolara di Sopra 50, Ozzano dell'Emilia 40064 BO, Italy). Sows were transferred to the experimental facility 1 month prior the expected delivery date and moved to the farrowing pen one week before. Animals were fed a standard pellet formula specifically made for breeding animals, produced by a local vendor (Molini Popolari Riuniti, Ellera-Umbertide 6019 PG, Italy). Drinking water was provided ad libitum, while the daily feed ratio was divided into two portions: early morning and afternoon. Light/dark cycle was set at 12/12h with a min of 40 Lux during light hours. Temperature was set at 21 ±1°C to meet sows thermal needs. With regards to piglet, two heat lamps were placed in dedicated areas of the farrowing crate to reach 32 ±1°C. For this study were used only animals previously enrolled in an experimental protocol approved by the Local Ethics Committee and the Italian Ministry of Health (Legislative Decree 26/2014, authorization n° 32/2021-PR, protocol number 2216A.17). The above-mentioned experimental protocol already included samplings on sows and piglets.
Briefly, fecal samples from piglets were collected, processed and used as the representation of the piglets' colon microbiota to undergo colonic short-term *in vitro* batch fermentation of sows' milk with different residues of amoxicillin in comparison to another antibiotic free milk sample.

12.2.2. Fecal piglets

Fecal samples were obtained from four 7 days old piglets, maintained refrigerated and processed within few hours. The fecal slurry was prepared by homogenizing 6 g of pooled feces (2 g of each piglet) in 54 mL of pre-reduced phosphate-buffered saline (PBS).

12.2.3. Sows milk (treatment and control samples)

Amoxicillin (Clamoxyl® RTU, Pfizer) was administered to sows, SID at 7 mg/kg IM from the second week of lactation until weaning (day 28). Milk samplings were manually obtained at different timepoints, after a prior administration of exogenous oxytocin, and immediately frozen (-80 °C) to preserve amoxicillin and its metabolites. Three kinds of milk employed in the *in vitro* fermentation

experiments were obtained from two pluriparous conventional adult sows aged two and three years approximately. Amox07 and amox08 are milk samplings from the first sow, with different concentrations of amoxicillin, collected 24 h and 2 h post intramuscular administration respectively; 9 days from the onset of lactation. Amox02 is the milk sample with no amoxicillin residues from the second lactating sow, used as the positive control and collected 6 days post-parturition. The blank control was instead used as a negative control. Milk samples were stored at -80 °C and analyzed at the bioanalytical laboratory of BioNotus® (Galileilaan 15, Niel 2845, Belgium) using a validated Liquid Chromatography–Mass Spectrometry (LC-MS/MS) method (BioNotus Method: MT-500A). Analyses were performed using Shimadzu Nexera X2 UHPLC, coupled with Shimadzu LC-MS 8050 system (Shimadzu, Kyoto, Japan). The data was acquired and processed via LabSolutions version 6.81 software. The lower and upper limit of quantification of amoxicillin were 10 ng/mL and 10000 ng/mL respectively.

12.2.4. Fecal batch-culture fermentation and samples collection

Colonic fermentations were conducted for 24 hours in independent vessels on 1 % (w/v) of amox02, on 1 % (w/v) of amox07, on 1 % (w/v) of amox08 (positive control), and on a blank control (BC) (negative control), using the in vitro gut model MICODE (Multi-Unit in vitro Colon Model), obtained by the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by Lucullus PIMS software (Applikon Biotechnology BV, NL) (Nissen et al., 2021a, 2021b). The preparation of the experiments was made according to published procedures (Koutsos et al., 2017; Nissen et al., 2021a, 2021b; Venardou et al., 2021; Wang et al., 2020; Deschamps et al., 2020). In details, bioreactors were autoclaved at 121 °C and 100 kPa for 15 min and once cooled aseptically filled with 90 mL of anaerobic pre-sterilized Fermentation Medium (FM) (Venardou et al., 2021). FM contained (per liter): 5 g/L yeast extract, 10 g/L ascorbic acid, 10 g/L sodium acetate, 5 g/L $(NH_4)_2SO_4$, 2 g/L urea, 0.2 g/L $MgSO_47H_2O$, 0.01 g/L $FeSO_47H_2O$, 0.007 g/L $MnSO_4xH_2O$, 0.01 g/L NaCl, 1 ml/L Tween 80, 0.05 g/L hemin and 0.5 g/L L-cysteine hydrochloride. The pH was adjusted to 7.0. Fermentation vessels were filled aseptically with 90 mL of FM and the bioreactor headplates were mounted, including previously sterilized and calibrated sensors, i.e. pH and DO_2 (Dissolved Oxygen) sensors (AppliSense, Applikon Biotechnology BV, NL). Anaerobic condition (0.0 – 0.1% w/v of DO_2) in each bioreactor was obtained in about 30 min flushing with filtered O_2-free N_2 through the mounted-in sparger of Minibio reactors (Applikon Biotechnology BV, NL), and was constantly kept over the experiment. Temperature was set at 39 °C and stirring at 100 rpm, while pH was adjusted to 7.0 and kept throughout the experiment with the automatic addition of filtered

NaOH or HCI (0.5 M). Once the exact environmental settings were reached, each of the four vessels was aseptically injected with 10 mL of pooled fecal slurry (10% w/v of pooled piglets' feces to a final concentration of 1%, w/v) and then three of them independently with 1 mL of amox02, amox07 or amox08 (to a final concentration of 1%, w/v), while the fourth vessel was set as blank control (BC, basal medium and 1% fecal slurry only). Batch cultures were run under these controlled conditions for a period of 26.10 h during which samples were collected at 3 time points (BL = Baseline, T1 = 18 h and EP = 24 h). The baseline (BL) was defined on the first pH changes (Venema, 2015) detected by Lucullus (1 read/10 s) via the pH Sensors of MICODE (AppliSense Sensors, Applikon Biotechnology BV, NL). For this work, the BL was set after 2.10 ± 0.28 h. Sampling was performed with a dedicated double syringe filtered system (Applikon Biotechnology BV, NL) connected to a float drawing from the bottom of the vessels without perturbing or interacting with the bioreactor's ecosystem. To guarantee a close control, monitoring and recording of fermentation parameters the software Lucullus 3.1 (PIMS, Applikon Biotechnology BV, NL) was used. This also allowed to keep the stability of all settings during the experiment. Fermentations were conducted in duplicate independent experiments, using for the first the fresh pooled slurry in pre-reduced PBS and for the second the same pooled slurry in pre-reduced PBS and 15% glycerol, previously stored at -80 °C for a week (Asare et al., 2021).

12.2.5. Experimental set up and pipeline of activities

Parallel and independent vessels for amox02, amox07, amox08, and blank control were run for 24 h after the adaptation of the fecal inoculum, defined as the baseline (BL). The entire experiment consisted of 9 duplicated biological cases (n = 18), including 4 theses (amox02, amox07, amox08, and blank control) and 3 time points (BL = 2.10 h, T1 = 18 h, and EP = 24 h) in duplicate. Samples of the different time points were used for qPCR and SPME GC-MS analyses. Pooled samples at the BL and the endpoints (EPs) of the 4 fermentation theses were used for 16S-r-DNA MiSeq analyses. After sterile sampling of 6 mL of bioreactor contents, samples were centrifuged at 16000 × g for 7 min to separate the pellets and the supernatants, which were used for bacterial DNA extraction and SPME-GC-MS analysis, respectively (Nissen et al., 2021a, 2021b). Specifically, microbial DNA extraction was conducted just after sampling so as not to reduce Firmicutes content (Nissen et al., 2021a, 2021b). DNA samples and SPME-GC-MS samples were then stored at -80 °C. Technical replicas of analyses were conducted in duplicate for SPME GC-MS (n = 36), in triplicate for qPCR (n = 54), and in single pooled cases (n = 5) for MiSeq.

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12.2.6. Microbiomics

12.2.6.1. *DNA extraction*

Bacterial DNA was extracted from the MICODE eluates at each time points, just after sampling; at the baseline (BL, when the fecal inoculum adapted to the *in vitro* condition), at the intermediate time point (T1, after 18 h), and at the endpoint (EP, after 24 h) using the Purelink Microbiome DNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). Bacterial DNA was extracted also from frozen sow's milk using the NucleoSpin Food DNA Isolation Kit (Macherey-Nagel, Duren, De). Nucleic acid purity and concentration was tested on BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK).

12.2.6.2. *DNA amplification and sequencing by Illumina MiSeq*

Samples from the BL, and the EP were used for MiSeq sequencing (Illumina Inc, San Diego, CA, USA). Bacterial diversity was obtained by the library preparation and sequencing of the 16S rRNA gene. The following two amplification steps were performed: an initial PCR amplification using 16S locus-specific PCR primers (16S-341F 5′-CCTACGGGNGGCWGCAG-3′ and 16S-805R 5′-GACTACHVGGGTATCTAATCC-3′) and a subsequent amplification integrating relevant flow-cell-binding domains (5′-TCGTCG GCAGCGTCAGATGTGTATAAGAGACAG-3′ for the forward primer and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ for the reverse overhang), and lastly unique indices selected among those available Nextera XT Index Kits were combined according to manufacturer's instructions (Illumina Inc, USA). Both input and final libraries were quantified by Qubit 2.0 Fluorometer (Invitrogen, USA). In addition, libraries were quality-tested by Agilent 2100 Bioanalyzer High Sensitivity DNA assay (Agilent technologies, Santa Clara, CA, USA). Libraries were sequenced in a MiSeq (Illumina Inc, USA) in the paired end with 300-bp read length (Marino et al., 2019). Sequencing was conducted by IGA Technology Service Srl (Udine, Italy).

12.2.6.3.*Sequence data analysis*

Reads were de-multiplexed based on Illumina indexing system. Sequences were analyzed using QIIME 1.5.0 (Caporaso et al., 2010). After filtering based on read quality and length (minimum quality = 25 and minimum length = 200), Operational Taxonomic Units (OTUs) defined by a 97% of similarity were picked using the Uclust v1.2.22 q method (Edgar, 2010) and the representative sequences were submitted to the RDP classifier (Wang et al., 2007) to obtain the taxonomy

assignment and the relative abundance of each OTU using the Greengenes 16S rRNA gene database (McDonald et al., 2012). Alpha- and beta-diversity analyses were performed using QIIME 1.5.0.

12.2.6.4. *Absolute enumeration of bacterial groups by qPCR*

Enumeration of bacterial groups was made by qPCR to quantify the microbiota at the BL and evidence changes after fermentation (Tamargo et al., 2022; Tanner et al., 2014; Tsitko et al., 2019; Westfall et al., 2018) and from the milk samples to quantify the bacterial loads, following previous protocols (Modesto et al., 2011; Nissen et al., 2021a, 2021b). For milk samples, 8 bacterial taxa were analyzed, namely Eubacteria, *Firmicutes*, *Lactobacillales*, *Bifidobacteriaceae*, *Enterobacteriaceae*, *Clostridium* group I, *Clostridium* group IV, *Escherichia coli*. For colonic fermentation samples, the previous 8 and other 6 taxa were analyzed, namely *Bacteroidetes*, *Bacteroides-Prevotella-Porphyromonas* (BPP) group, *Atopobium-Collinsella-Eggerthella* (ATOP) group, *Bifidobacterium longum*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*) (Table S1) were assessed by qPCR on a QuantStudio 5 System (Applied Biosystem, Thermo Fisher, USA).

12.2.7. Metabolomics

12.2.7.1. Volatilome analysis

Volatile organic compounds (VOCs) evaluation was performed on a Gas Chromatograph (7890A , Agilent, Santa Clara, CA, USA) Mass Spectrometer (5975, Agilent, USA) operating in the electron impact mode (ionization voltage of 70 eV) equipped with a Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm ID) (Chrompack, Middelburg, The Netherlands). The SPME (Solid Phase Micro-Extraction) GC-MS protocol and the identification of volatile compounds were done accordingly to previous reports, with minor modifications (Nissen et al., 2021; Guerzoni et al., 2007; Di Cagno et al., 2011; Casciano et al., 2021). Preconditioning, absorption, desorption of SPME–GC-MS analysis, and all data processing were carried out according to literature (Nissen et al., 2021; Di Cagno et al., 2011; Casciano et al., 2021). Molecules Identification was carried out by searching mass spectra in the NIST 11 MSMS library (NIST, Gaithersburg, MD, USA). The VOCs were then sorted and super-normalized for respective chemical class, i.e., organic acids, alcohols, and other VOCs (Nissen et al., 2021a). In samples at BL the main microbial metabolites related to fermentation of foods were absolutely quantified in mM by SPME GC-MS and an internal standard (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Nissen et al., 2021a; Di Cagno et al., 2011; Casciano et al., 2021).

12.2.7.2. *Shift of main microbial VOCs*

In samples prior to *in vitro* colonic fermentation (BL) (Table S2) the main microbial metabolites related to fermentation of foods were also absolutely quantified in mg/kg with the aforementioned SPME GC-MS approach and the internal standard, but with different cutoffs (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg) (Casciano et al., 2021; Di Cagno et al., 2011; Nissen et al., 2021a). For these compounds, samples at T1 and EP were compared to the BL and values were expressed as shifts. Values were computed as follows; i) each single compound was normalized (mean centering method) within its dataset, which included cases from amox02, amox07, and amox08, and the blank control at different time points; ii) the BL dataset (Table S2) was then subtracted to the fermentation time points; iii) post-hoc analysis was done to compare the sample productions of a single molecule.

12.2.8. Data processing and statistical analysis

For metabolomics, one-way ANOVA model ($p < 0.05$) was used to determine significant VOCs among the raw data of peak's area of the GC-MS chromatograms. The significant VOCs (n = 65) representing the total volatilome of the experiments were analyzed differently; i) 8 main VOCs related to microbial fermentation of foods were absolutely quantified and normalized and their BL values were subtracted from T1 and EP values and represented as blox plots, including post hoc Tukey HSD test ($p < 0.05$); ii) the remaining volatilome was relatively quantified, sorted for main chemical classes and super-normalized, then each dataset was computed for Principal Component Analysis (PCA) to distribute the results on a plane and coupled to Multivariate ANOVA (MANOVA) ($p < 0.01$) to address specific contributes by categorical predictors.

For the sequencing data analysis, the QIIME pipeline version 1.5.0 was used. Within-community diversity (alpha diversity) was calculated using observed OTUs, Chao1 Shannon, Simpson, and Good's coverage indexes with 10 sampling repetitions at each sampling depth. Student's t-test was applied to compare the latest sequence/sample values of different treatments within an index. Analysis of similarity (ANOSIM) and the ADONIS test were used to determine statistical differences between samples (beta diversity) following the QIIME compare_categories.py script and using weighted and unweighted phylogenetic UniFrac distance matrices. Principal Coordinate Analysis (PCoA) plots were generated using the QIIME beta diversity plots workflow (Marino et al., 2019).

For microbiomics, ANOVA model for group comparison (BL versus EPs) ($p < 0.05$) was performed for MiSeq and MANOVA ($p < 0.05$) model (categorized for the time points and the treatments) was performed for qPCR. Afterwards, the significant variables and others of peculiar interest were selected and the shifts in abundance were calculated as $Log_2(F/C)$ (Love et al., 2014). Then, *post hoc*

Tukey HSD test on the raw data ($p < 0.05$) was performed to define differences among treatments (MiSeq and qPCR) or time points (qPCR). The baselines of values for the volatilome and for the microbiota were that obtained sampling just after adaptation of the microbiota to the bioreactor condition (Nissen et al., 2021b). Normalization of datasets was performed with the mean centering method. Statistics and graphics were made with Statistica v.8.0 (Tibco, Palo Alto, CA, USA).

12.3. Results and discussion

It was reported that early-life in-feed antibiotic treatments could alter the gut microbiota of young piglets, affecting digestive physiology, with greater respect to carbohydrates metabolism (Lin et al., 2018; Mu et al., 2017) and future growth (Yu et al., 2018). Indeed, once ingested, amoxicillin undergoes acid-catalyzed degradation in the stomach and enzymatic degradation by intestinal flora; previous studies showed the presence of various beta-lactamase enzymes in the normal intestinal microbiota of juvenile pigs (Reyns et al., 2008). Being amoxicillin a hydrophilic drug, it can be mainly found in the liquid fraction once ingested through milk (Ozdemir et al., 2018); however, using a gut model adapted to suckling piglets' colon microbiota represents a valuable approach to study gut microbiota shift and their metabolites as a consequence of milk amoxicillin residues absorption.

12.3.1. Amoxicillin LC-MS/MS quantifications

Amox07 milk sample was collected 24 h post administration; amoxicillin was found Below Limit of Quantification (ie <10 ng/mL). Instead, amox08, collected 2 h post maternal administration, was quantified as 32.741 ng amoxicillin/mL. Amox02 was not analyzed as the sow was never treated with amoxicillin; this sample was used as positive control.

12.3.2. Microbiomics

12.3.2.1. Analysis of the biodiversity in the microbiota by relative quantification of 16S-rDNA

The microbiota diversity indices were analyzed both to study the impact of different amoxicillin residues in the sow's milk on microbial population of piglets' colon and to assess population's stability during fermentation of the different bioreactors (Figure S1). The BL value (as defined by first pH decrease) was compared to the EPs of fermentation of different treatments.
Considering richness, it is unquestionable that an increase (Observed OTUs) cannot happen during *in vitro* fermentation (Isenring et al., 2021), and reductions in respect to the BL were significantly different just for EPs of amox02 and amox07.

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A reduction in abundance index (Chao 1) from the BL to the EP was recorded for amox02 and amox07, while amox08 scored a slight increase, although significant differences were just that of the highest values (amox08) in respect to the lowest values (amox07).

Significant reduction in evenness (Shannon) from the BL to the EP were seen for any substrate, and different values were recorded at the EP of amox08 in respect to the lowest values of amox07. This latter feature could be a first clue to a possible perturbation of microbiota eubiosis.

Reductions in dominance (Simpson) were seen from the BL to the EP for any substrate, but significantly just for amox07. This latter feature could be ascribed to the reduction at the EP of a dominant phylum.

Additionally, the Good's Index, relative to rare OTUs, was kept similar from the BL to the EPs of any milk substrate with just slight reductions, but no significative differences. This feature means that the stability of MICODE environment was maintained throughout the entire experimental period, because the rare taxa, which need strict ecological conditions, were still present at the EPs.

Overall, from the alpha diversity analyses the resulting scenario indicates that generally the eubiosis in respect to the BL was maintained after colonic milk fermentations, except for slight reductions in evenness and dominance by amox08.

When the bacterial diversity between samples (beta diversity) was examined with Bray–Curtis analysis, the pooled sample relative to the BL was set not so much distant, although discriminated in respect to the samples at the EP of fermentation, as demonstrated by principal coordinate analysis (PCoA) based on an unweighted (qualitative) phylogenetic UniFrac distance matrix. Beta diversity indicated that the shifts happened in the microbiota of piglets after milk fermentation were not so dramatic and overall the differences among samples from colonic fermentations were limited.

12.3.2.2. Analysis of the shift in the phyla of microbiota by relative quantification of 16S-rDNA

Results from microbiota analyses at the phylum level (Table 1) have defined that the core microbiota of any sample was ruled by two main phyla with relative abundance higher than 10%, and three minors with relative abundance lower than 10%. *Firmicutes* and *Bacteroidetes*, accounted for almost the 80% of the whole pie, while *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* accounted for the remaining. In any fermentation sample *Firmicutes* and *Bacteroidetes* were reduced in respect to the BL, although not significantly. *Actinobacteria* were reduced significantly in any milk fermentations, while *Fusobacteria* and *Proteobacteria* were increased, but significantly just for the latter. The unaffected changes of the core microbiota make us generally believe that an equilibrium among such wide taxa was maintained even after fermentation.

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Table 1. Shifts of the microbiota at the phylum level from 16S-rDNA sequencing

#OTU ID	% R.Q.	Log_2(F/C)			ANOVA*
	Baseline	amox02	amox07	amox08	*p* value
Euryarchaeota	0.01[a]	-2.50[b]	-3.03[b]	-2.56[b]	0.001626
Bacteria;Other	0.04[a]	-2.00[b]	-1.99[b]	-1.35[b]	0.017493
Actinobacteria	2.71[a]	-2.95[b]	-2.64[b]	-1.59[b]	0.024128
Bacteroidetes	21.66	-1.18	-1.08	-2.56	0.080744
Firmicutes	61.69	-0.81	-0.41	-0.32	0.173732
Fusobacteria	8.12[a]	2.09[b]	1.91[b]	1.99[b]	0.009156
Proteobacteria	5.75	1.83	1.11	1.24	0.189654
Synergistetes	0.02[a]	-3.76[b]	0.00[a]	-3.24[b]	0.004404

*One-way ANOVA with $p < 0.05$. R.Q. = Relative Quantity. [abc] Letters indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$).

12.3.2.3. Analysis of the shift in the families of microbiota by relative quantification of 16S-rDNA

Results from the microbiota analysis at the family level (Table 2) evidenced a scenario discriminated by the fermentation and seldom by the severity of amoxicillin concentration.

Table 2. Shifts of the microbiota at the family level from 16S-rDNA sequencing

#OTU ID	% R.Q.	Log_2(F/C)			ANOVA*
	M11 BL	amox02	amox07	amox08	*p* value
Actinomycetaceae	2.55[a]	-3.11[b]	-2.61[b]	-1.61[b]	0.024667
Bifidobacteriaceae	0.06 [a]	-2.34[b]	-3.10[b]	-3.89[b]	0.001018
Bacteroidaceae	18.48	-0.99	-0.88	-2.41	0.120363
Porphyromonadaceae	1.30[a]	-3.04[b]	-3.11[b]	-4.22[b]	0.002328
Prevotellaceae	0.83[a]	-5.71[b]	-5.24[b]	-5.85[b]	0.000033
Rikenellaceae	0.26[a]	-7.31[b]	-7.84[b]	-6.78[b]	0.000008
Sphingobacteriaceae	0.72[a]	-3.74[b]	-3.86[b]	-2.91[b]	0.002006
Staphylococcaceae	0.01[b]	1.34[a]	1.19[a]	0.42[ab]	0.000149
Enterococcaceae	0.17[b]	5.48[a]	5.86[b]	6.43[b]	0.027770
Lactobacillaceae	34.52[a]	-1.89[b]	-3.14[b]	-2.19[b]	0.010708
Streptococcaceae	0.93[a]	-2.08[b]	-2.45[b]	-1.51[b]	0.017605
Clostridiales;Other	0.09[a]	-3.73[b]	-5.26[b]	-4.79[b]	0.000991
Clostridiaceae	2.44	1.84	3.52	2.21	0.057749
Lachnospiraceae	9.30[a]	-2.18[b]	-2.59[b]	-2.29[b]	0.001559
Peptococcaceae	0.66	-0.53	-0.84	-0.60	0.096887
Peptostreptococcaceae	2.20[a]	-0.75[b]	-1.55[c]	0.25[b]	0.000012
Ruminococcaceae	7.04[a]	-2.73[b]	-1.37[b]	-2.93[b]	0.002811
Veillonellaceae	2.13	1.19	-1.85	1.91	0.638170
Coriobacteriaceae	0.34	-1.02	0.61	0.66	0.805605

Coprobacillaceae	0.26[b]	0.00[b]	-0.05[b]	1.68[a]	0.000092
Erysipelotrichaceae	1.52	-1.84	-2.51	-1.76	0.009737
Fusobacteriaceae	8.12[a]	2.09[b]	1.91[b]	1.99[b]	0.009156
Alcaligenaceae	0.26[a]	-0.39[a]	-2.12[b]	-1.53[b]	0.045315
Desulfovibrionaceae	0.66	-4.72	-5.25	-5.17	0.000032
Campylobacteraceae	0.01	1.83	2.17	1.50	0.113515
Enterobacteriaceae	4.40	2.15	1.38	1.19	0.256852
Pasteurellaceae	0.08[b]	3.01[a]	3.42[a]	5.46[a]	0.023245

*One-way ANOVA with $p < 0.05$. R.Q. = Relative Quantity. [abc] Letters indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$).

Indeed, amox08 during colonic fermentation was able to reduce the content of opportunistic *Porhyromonadaceae* and limit the growth of *Staphylococcaceae*, *Enterobacteriaceae* and *Desulfovibrionaceae* in a significative difference in respect to the milk control with no antibiotic residues (amox02). For the intestinal health of piglets, the capability to reduce the content of opportunistic and pathogens, such as those included in the families *Staphylococcaceae*, *Enterobacteriaceae*, and *Desulfovibrionaceae*, is an important goal, because these bacterial taxa are culprits of dysbiosis induction and can led to intestinal pathogenesis (Gresse et al., 2017; Hasan et al., 2018). For example, the first family is generally transferred to the piglets' colon from the batch flora of the mammary glands and some species are associated with several important piglets' pathologies (Wang et al., 2017).

Oppositely, the antibiotic residues exerted an undesired effect towards important beneficial taxa of the piglets' colon microbiota, due to a wider range of targets. This effect was different in respect to the different capacity of a taxon to generically resist to insults. In particular, this effect was dramatically high in sensitive *Bifidobacteriaceae*, that were almost depleted after amox08 fermentation, and in sensitive *Ruminococcaceae*, that were reduced of almost three-folds, in respect to the BL and two time more than the milk without antibiotic residues. Also, this effect was observed in dominant *Lactobacillaceae*. For the intestinal health of piglets the *Lactobacillales* order is fundamental. Since the first days the piglets' colon microbiota is dominated by *Lactobacillaceae* mainly, accounting for a third of the whole pie (Petri et al., 2010). This taxon is inherited from the sow milk, that in our milk samples had a mean value of 2.01E+05 cells/mL, and establish an important symbiosis up to the adult phase, contributing to the microbiota's beneficial effects (Petri et al., 2010). Additionally, several lactobacilli strains of pig's origin were proposed as probiotic and porcine feed additive, e.g. *L. salivarius* LS6 (Yeo et al., 2016). However, in our work this community was reduced by the action of antibiotic residue in milk.

Unexpectedly, the effect observed for *Lactobacillaceae* was observed also for important commensal fibrolytic bacteria, such as *Bacteroidaceae*, that was reduced of 2.4 folds in respect to the BL;

although not significantly. Furthermore, it is observed in some taxa a competitive advantage by the presence of antibiotic residues, recording an increased abundance. This phenomenon was particularly strong in those bacterial taxa phenotypically heterogeneous. For example, from the superior taxonomic level of *Lactobacillales*, two family behaved oppositely; as we have just said, the *Lactobacillaceae* were reduced (from 34.5% at the baseline to 7.6% at the endpoint of fermentation with amox08), but the *Enterococcaceae* were fostered (from 0.16% at the baseline to 14.5% at the endpoint of fermentation with amox08). Similarly, from the superior level of Gammaproteobacteria, the *Enterobacteriaceae* were more limited (from 4.4% at the baseline to 19.5% and 10.0% at the endpoint of amox02 and amox08 fermentations, respectively), but the *Pasteurellaceae* were increased (from 0.07% at the baseline to 0.6% and 3.3% at the endpoint of amox02 and amox08 fermentations, respectively).

For the intestinal health of piglets, the role of *Clostridiales* is crucial, because represents a large portion of the core microbiota, accounting generally for the 30% of the colon microbiota of piglets. The reduction of *C. perfringens*, *C. baratii*, and *C. frigidicarnis* is fine since are causative agents of enteritis in pigs and used to spread in herds, additionally toxigenic *C. perfringens* can lead to death, and also represent a risk for the consumers (Mehdizadeh Gohari et al., 2021). Also the increased abundance of *C. butyricum* has to be observed as a positive feature, since this taxon is a butyrate producer and has been proposed and successfully tested as a probiotic for weanling pigs feed (Casas et al., 2020; Peeters et al., 2019).

The inhibitory activity against commensals and beneficial taxa is clearly a side effect of wide range antibiotics, such as amoxicillin, that other than the opportunistic taxa also reduce largely the richness of the microbiota, including the reduction of beneficial bacteria.

We can summarize the presence of antibiotic residues in the milk can modulate the microbiota of piglets via four main actions. I) A desired inhibitory effect towards several opportunistic bacterial taxa; ii) an inhibitory effect towards sensitive commensal taxa; iii) a stimulation of tough (generally resistant to stress) bacterial taxa.

Considering the overall scenario there is evidence that some amoxicillin resistant taxa took advantage of the depletion of abundant opportunistic sensitive ones. For example, three are the cases encountered in our work: i) the split in the *Lactobacillales* class, where the *Enterococcaceae* took advantage from the depletion of *Lactobacillaceae*. *Enterococcaceae* are not harmful and has been proposed even as probiotics for post-weaning pigs (Sato et al., 2019), but can cause bacteremia, especially during antibiotic treatment, as actually it is reported in literature to be resistant to amoxicillin in poultry (Cuccato et al., 2021). ii) The split in the *Clostridiales* class, that deepened at

the lowest taxonomic level, was driven by amox08 fermentation. This taxon was reduced for its overall content, but the reduction was higher for the portion of the more sensitive taxa, than that of the tougher taxa. Among these former taxa, there are also some reported to be generally resistant to antibiotic and also specifically to amoxicillin as; *Peptostreptococcaceae* and the *Clostridiaceae(de Jong et al., 2014)*. iii) The split in Gammaproteobacteria order, described by the constrained growth of *Enterobacteriaceae,* and the rose in the abundance of *Pasteurellaceae.* Even in this situation the reduction of *Enterobacteriaceae* from our results is a positive feature to maintain a healthy colon of the animals, but *Pasteurellaceae* are important pathogens affecting the respiratory tract of pigs, that in the past were susceptible to antibiotic treatments (de Jong et al., 2014), but nowadays are becoming resistant developing specific phenotypes (Gao et al., 2021).

12.3.2.4. Analysis of the shift in the genera and species of microbiota by relative quantification of 16S-rDNA

In order to try to account the shift previously observed to some specific taxa, a relative quantification of 16S-rDNA was performed (Table 3). Specifically, the reduction of *Lactobacillaceae* in contrast to the increase in *Enterococcaceae* has been generated by some key players, as *L. crispatus* (from 9.7% at the baseline to 2.2% and 1.0% at the endpoint of amox02 and amox08 fermentations, respectively), *L. antrii* (from 8.3% at the baseline to 3.7% and 3.6% at the endpoint of amox02 and amox08 fermentations, respectively), *L. gasseri* (from 9.5% at the baseline to 1.6% and 1.4% at the endpoint of amox02 and amox08 fermentations, respectively) and *L. delbruecki* (from 1.5% at the baseline to 0.3% and 0.1% at the endpoint of amox02 and amox08 fermentations, respectively). Oppositely, under the *Enterococcaceae*, the species that were overrepresented were *Enterococcus durans* (from 0.1% at the baseline to 3.2% and 4.5% at the endpoint of amox02 and amox08 fermentations, respectively) and *E. faecalis* (from cutoff levels at the baseline to 3.7% and 9.0% at the endpoint of amox02 and amox08 fermentations, respectively).

Table 3. Shifts of the microbiota at the genus and species level from 16S-rDNA sequencing.

#OTU ID	% R.Q.	Log_2(F/C)			ANOVA*
	Baseline	amox02	amox07	amox08	*p* value
Methanobrevibacter	0.01^{a}	-2.50^{b}	-3.03^{b}	-2.56^{b}	0.001626
Actinomyces	2.54^{a}	-3.10^{b}	-2.60^{b}	-1.61^{b}	0.024542
Corynebacterium	0.02	-0.68	-2.21	-0.24	0.360138
Bifidobacterium	0.06^{a}	-3.34^{b}	-3.10^{b}	-3.89^{b}	0.001018
Bacteroides	18.48^{a}	-0.99^{b}	-0.88^{ab}	-2.41^{c}	0.017239
Porphyromonas	0.09^{a}	-4.50^{b}	-6.35^{b}	-5.29^{b}	0.000364
Parabacteroides	1.21^{a}	-2.97^{b}	-3.01^{b}	-4.17^{b}	0.002679

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Prevotella	0.83[a]	-5.71[b]	-5.24[b]	-5.85[b]	0.000033
Rikenella	0.26[a]	-7.31[b]	-7.84[b]	-6.78[b]	0.000008
Enterococcus	0.17	5.48	5.86	6.43	0.127750
Lactobacillus	34.52[a]	-1.99[b]	-3.14[b]	-2.19[b]	0.010708
Streptococcus	0.93[a]	-2.08[b]	-2.45[b]	-1.51[b]	0.017605
Clostridiaceae;Other	0.23[a]	-3.73[b]	-5.26[b]	-4.79[b]	0.000991
Clostridium	2.07	1.88	3.52	1.74	0.434835
Finegoldia	0.01	6.09	8.24	7.94	0.284612
Mogibacterium	0.21[a]	-4.52[b]	-4.19[b]	-3.29[b]	0.001492
Lachnospiraceae;Other	2.61[a]	-1.74[b]	-3.10[b]	-2.22[b]	0.017499
Blautia	0.03	-1.92	-0.59	-0.74	0.185554
Dorea	1.02[a]	-3.11[b]	-4.92[b]	-3.22[b]	0.003279
Roseburia	0.01	-0.44	-0.70	-0.24	0.189321
Ruminococcus	5.59[a]	-2.41[b]	-3.38[b]	-2.24[b]	0.007038
Peptococcus	0.66	-0.54	-0.84	-0.60	0.056183
Peptostreptococcaceae;Other	0.09	2.98	2.14	4.27	0.399730
Clostridium	0.31[a]	-1.73[bc]	-2.28[c]	-1.08[b]	0.049276
Peptostreptococcus	1.79[a]	-1.92[b]	-2.73[b]	-1.45[b]	0.026758
Faecalibacterium	0.47[a]	-2.92[b]	-3.20[b]	-2.55[b]	0.001763
Oscillospira	1.77[a]	-3.06[b]	-3.31[b]	-1.69[b]	0.025503
Ruminococcus	4.73[a]	-4.32[b]	-5.51[b]	-4.12[b]	0.000510
Megasphaera	0.06[a]	-3.74[b]	-5.59[b]	-3.12[b]	0.003442
Negativicoccus	1.10	2.07	-0.97	2.83	0.522025
Phascolarctobacterium	0.96[a]	-2.34[b]	-6.12[c]	-2.64[b]	0.016017
Veillonella	0.02	2.05	-2.12	0.09	0.765288
Atopobium	0.02[a]	-2.50[bc]	-4.03[c]	-1.75[b]	0.026410
Collinsella	0.01	-1.07	-1.82	0.15	0.530060
Eggerthella	0.06	-1.13	-0.18	0.78	0.983175
Coprobacillus	0.25	0.01	-0.05	1.69	0.672057
Bulleidia	1.01[a]	-1.83[b]	-3.47[b]	-1.66[b]	0.031552
Eubacterium	0.49[a]	-1.95[b]	-1.51[b]	-2.18[b]	0.011357
Fusobacterium	8.12[a]	2.09[b]	1.91[b]	1.99[b]	0.009146
Sutterella	0.26	-0.39	-2.37	-1.56	0.237870
Bilophila	0.03[a]	-3.26[b]	-2.21[b]	-2.15[b]	0.008941
Desulfovibrio	0.63[a]	-6.10[b]	-5.92[b]	-5.81[b]	0.000004
Escherichia	4.39	1.99	1.24	1.15	0.240319
Aggregatibacter	0.06	3.45	3.87	5.91	0.475903
Pseudomonas	0.05	-1.34	-0.65	-1.47	0.091075
Methanobrevibacter;s__smithii	0.01[a]	-2.50[b]	-3.03[b]	-2.56[b]	0.001626
Bacteroides;s__acidifaciens	0.04[a]	-2.62[b]	-3.95[b]	-3.48[b]	0.004352
Bacteroides;s__heparinolyticus	0.23[a]	-2.92[b]	-3.23[b]	-2.27[b]	0.004990
Bacteroides;s__ovatus	0.37[a]	-2.58[b]	-4.41[b]	-3.45[b]	0.006093
Bacteroides;s__pyogenes	4.18[a]	1.00[b]	1.09[b]	-1.72[c]	0.025060
Bacteroides;s__uniformis	0.20[a]	-1.71[b]	-2.38[b]	-4.30[c]	0.031170
Bacteroides;s__vulgatus	1.26[a]	-2.37[b]	-2.46[b]	-1.61[b]	0.014477
Parabacteroides;s__distasonis	1.12[a]	-3.05[b]	-3.07[b]	-4.17[b]	0.002280

Prevotella;s__	0.64[a]	-6.11[b]	-5.93[b]	-6.46[b]	0.000009
Enterococcus;s__durans	0.13[b]	4.69[a]	5.14[a]	5.13[a]	0.038408
Enterococcus;s__faecalis	0.03	6.84	7.18	8.13	0.220472
Lactobacillus;s__antri	8.33[a]	-1.20[b]	-1.68[b]	-1.15[b]	0.021077
Lactobacillus;s__crispatus	9.75[q]	-2.13[b]	-5.85[c]	-3.19[bc]	0.018745
Lactobacillus;s__delbrueckii	1.50[a]	-2.07[b]	-5.76[c]	-3.18[bc]	0.020505
Lactobacillus;s__gasseri	9.54[a]	-2.57[b]	-4.74[b]	-2.75[b]	0.008456
Streptococcus;s__hyointestinalis	0.33[a]	-2.73[b]	-3.1 [b]	-2.43[b]	0.002672
Clostridiaceae;Other;Other	0.14[a]	-3.34[b]	-2.12[b]	-2.12[b]	0.011455
Clostridium;s__baratii	0.02	-0.32	-0.48	-0.38	0.054278
Clostridium;s__butyricum	0.01	11.77	13.60	11.35	0.427891
Clostridium;s__cadaveris	0.01	2.85	1.56	4.72	0.059228
Clostridium;s__frigidicarnis	0.01[a]	-3.63[b]	-3.65[b]	-2.69[b]	0.003019
Clostridium;s__perfringens	1.90	-1.49	-2.57	-0.44	0.226359
Finegoldia;s__magna	0.02	4.35	6.71	6.76	0.313308
Dorea;s__	1.01[a]	-3.11[b]	-4.97[b]	-3.22[b]	0.003398
Roseburia;s__faecis	0.02[a]	-1.17[b]	-0.70[ab]	-1.24[b]	0.001921
Ruminococcus;s__	3.12[a]	-3.28[b]	-4.35[b]	-3.14[b]	0.021094
Ruminococcus;s__gnavus	2.47[a]	-1.76[b]	-2.69[b]	-1.58[b]	0.047608
Faecalibacterium;s__	0.45[a]	-3.12[b]	-3.35[b]	-2.67[b]	0.029017
Faecalibacterium;s__prausnitzii	0.02[a]	-0.91[ab]	-1.44[b]	-1.10[b]	0.025503
Negativicoccus;s__succinicivorans	1.10[b]	2.07[a]	-0.97[c]	2.83[a]	0.015768
Veillonella;Other	0.02[c]	4.24[a]	0.30[c]	1.64[b]	0.018016
Adlercreutzia;s__	0.17[b]	-0.53[b]	1.38[a]	1.17[a]	0.026410
Eggerthella;s__lenta	0.06	-1.13	-0.18	0.78	0.135460
Coprobacillus;s__cateniformis	0.25[b]	0.02[b]	-0.03[b]	1.69[a]	0.031552
Bulleidia;s__	1.01[a]	-1.83[b]	-3.47[c]	-1.66[b]	0.005019
Fusobacterium;s__gonidiaformans	1.45[b]	4.54[a]	4.36[a]	4.45[a]	0.000031
Sutterella;s__parvirubra	0.02[c]	3.44[a]	1.41[b]	2.20[ab]	0.008941
Escherichia;Other	4.23	1.99	1.25	1.14	0.266914
Escherichia;s__	0.04	1.74	1.20	0.78	0.222009
Escherichia;s__albertii	0.11	1.98	1.17	1.29	0.475903
Actinobacillus;s__porcinus	0.02[a]	-3.05[b]	-4.70[b]	-4.69[b]	0.003434
Acinetobacter;s__lwoffii	0.19[a]	-6.22[c]	-6.28[c]	-1.93[b]	0.031993

*One-way ANOVA with $p < 0.05$. R.Q. = Relative Quantity. [abc] Letters indicate significant differences within a line by Tukey's honestly significant differences (HSD) test ($p < 0.05$).

For the intestinal health of piglets, the role of *Clostridiales* is crucial, because represents a large portion of the core microbiota (Yang et al., 2021). Actually, in our samples accounted for about the 24% of total microbiota at the baseline. They include some pathogen targets of amoxicillin, but others are commensals butyrate producers. For example, while the stress sensitive *Lachnospiraceae* or the *Ruminococcaceae* were around 10-fold inhibited in amox08 colon fermented microbiota in comparison to the control, with the same milk the opportunistic *Veillonaceae* and *Peptostreptococcaceae* slighly increased and even more in *Clostridiaceae* (5-fold). In particular,

within this latter family, another phenotypical split had happened, indeed even if all the three major genera of this family were fostered by any milk substrate, the sole genus *Clostridium* grew less than the control (7.6% and 6.9% at the endpoint of amox08 and amox02 fermentations, respectively), while genra *Finegoldia* (0.7% and 2.5% at the endpoint of amox02 and amox08 fermentations, respectively) and *Anaerococcus* (0.3% and 1.7% at the endpoint of amox02 and amox08 fermentations, respectively) were increased much more than the control. Noteworthy, even deeper in the genus *Clostridium*, some species were limited while others were fostered after fermentation with amox08. For example, the harmful *C. perfringens* (from 1.9% at the baseline to 1.4% at the endpoint), *C. baratii* and *C. frigidicarnis* were underrepresented, while *C. butyricum* and *C. cadaveris* were overrepresented.

12.3.2.5. *Absolute enumeration of selected taxa of milk*

We firstly considered milk microbiota to give a more complete picture of all the ecological factors affecting microbial shift in MICODE gut model. For a robust description of the core microbiota and its shifts produced after fermentation of the different milk samples, we performed qPCR absolute quantifications of 10 selected targets related to healthy piglets' colon ecology, either at top or low taxonomic levels. We have also considered the bacterial loads of 8 principal bacterial taxa common in sow's milk. Considering milk, generally there were significant differences mainly comparing the milk samples with no antibiotic residues (amox02) or the milk samples with the lowest antibiotic residues (amox07) to the milk samples with antibiotic residues (amox08). In the milk samples, total bacterial load accounted for a mean of 1.12E+06 cells/mL and amox08 had 44% significantly less abundance than the milk with no antibiotic residues. *Firmicutes* content had a mean value of 2.78E+05 and amox08 had 40% significantly less abundance than amox02. *Lactobacillales* content had a mean value of 2.01E+05 and amox08 had 40% and 33% significantly less abundance than amox02 and amox07, respectively. *Clostridium* group I and *Clostridium* group IV had means values of 1.91E+04 and 2.59E+04, and amox08 had 32% and 46% significantly less abundance than amox02, respectively. *Enterobacteriaceae* had a mean value of 1.41E+04 and amox08 had 34% significantly less abundance than amox02. In this family *Escherichia coli* was detectable just in the amox02 and amox07 samples, accounting for a mean value of 1.68E+02. A similar outcome was also seen for the content of *Bifidobacteriaceae* that was detectable just in the amox02 and amox07 samples, accounting for a mean value of 1.8E+04. From these results it is possible to summarize that the presence of amoxicillin residues in the milk diminished depending on concentration its indigenous microflora. Overall, in milk samples the presence of amoxicillin resulted in lower bacterial loads, that

desirably were relative to reduction of opportunistic bacteria, but also and undesirably to commensals *Lactobacillales* and to depletion of *Bifidobacteriaceae*. These loads of exogenous microbes should not have impacted on the colon microbiota, because are at least 1000000 times lower than what was quantified at the baseline of fermentation.

12.2.3.6. Absolute enumeration of selected taxa of colonic fermentation samples

With the same analytical approach, the shifts occurred during MICODE fermentation were considered. In general, significant differences were found for the milk substrates, but not for the blank control. At the BL the abundance similarly averaged (no significant differences among BL raw data) for 1.05E+10 and trended to increase, except for the blank control, with no significant differences. Considering the two main phyla, in fermentation samples *Firmicutes* and *Bacteroidetes* had opposite trends. The former was increased by amox02 and amox07 and reduced by amox08 (of about 2.62E+09 cells/mL), the latter was reduced by each milk samples, but not significantly for amox02. In particular, amox08 reduced *Bacteroidetes* of about 1.39E+09 cells/mL, that was circa 9 time more the reduction of amox02. In the taxon *Firmicutes*, the *Lactobacillales* recorded an increase for amox02 and significant reduction just for amox08, that was reduced almost thrice after fermentation and approximately 6 times more than amox07. The *Clostridium* group I was significantly reduced at the EP just by amox08 (-2.32 folds) and significantly increased with amox02 and amox07, of 1.31- and 1.43-folds, respectively. The *Clostridium* group IV was reduced by each treatment and significantly just by amox08, but the reduction scored by amox08 was almost thrice that of amox02. In the taxon *Bacteroidetes*, the BPP group quantified mainly the *Bacteroides* abundance, and recorded significant shifts in reduction for any milk sample, with amox08 having more than the double the strength of amox02. Considering the *Enterobacteriaceae* and the *E. coli* taxa, significant reductions from the BL on were observed just for the amox08 sample at the EP. Similarly, the *Bifidobacteriaceae* were significantly reduced just by amox08, but values under the detection limit were observed for amox07. In conclusion, just amox08 fermentation was able to contain and reduce opportunistic bacteria in piglets' colon, but also reduced the abundance of commensals and beneficials.

Table 4. Enumeration (cells/mL) by qPCR of core microbiota of milk and fermentation samples.

Eubacteria						
Sample	Cells/mL			Log_2(F/C)		MANOVA
	Milk ± SD*	BL raw**	BL mean ± SD	T1	EP	
amox02	1.80E+06 ± 1.50E+06^{A}	1.26E+10	1.05E+10 ± 1.92E+09^{b}	1.08^{aA}	0.26^{ab}	*0.014929*
amox07	1.23E+06 ± 1.12E+06^{A}	1.01E+10	1.05E+10 ± 1.92E+09	0.54^{AB}	0.31	*0.060255*
amox08	3.25E+05 ± 1.21E+05^{B}	8.84E+09	1.05E+10 ± 1.92E+09	0.19^{B}	0.26	*0.822842*

blank	n.a.	1.06E+10	1.05E+10 ± 1.92E+09	-0.43^{B}	-0.43	*0.088726*
	0.046181	*0.072272*		*0.034454*	*0.987142*	*p value*
Firmicutes						
amox02	4.16E+05 ± 2.76E+05	1.62E+09	2.73E+09 ± 9.73E+08^{b}	0.72^{abA}	1.20^{aaA}	*0.037431*
amox07	3.37E+05 ± 1.89E+05	3.17E+09	2.73E+09 ± 9.73E+08^{b}	1.28^{aA}	1.73^{aA}	*0.005016*
amox08	7.98E+04 ± 3.47E+04	3.41E+09	2.73E+09 ± 9.73E+08^{a}	-0.29^{aB}	-4.15^{bC}	*0.018042*
blank	n.a.	2.92E+09	2.73E+09 ± 9.73E+08	-0.43^{B}	0.29^{B}	*0.276141*
	0.061105	*0.076691*		*0.012030*	*0.000066*	*p value*
Bacteroidetes						
amox02	n.a.	1.37E+09	1.68E+09 ± 3.73E+08	-0.85^{A}	-0.16^{A}	*0.089309*
amox07	n.a.	1.58E+09	1.68E+09 ± 3.73E+08^{a}	-1.16^{bB}	-1.44^{bB}	*0.002010*
amox08	n.a.	2.10E+09	1.68E+09 ± 3.73E+08^{a}	-2.81^{bB}	-2.67^{bC}	*0.001914*
blank	n.a.	1.58E+09	1.68E+09 ± 3.73E+08^{a}	-0.43^{aA}	-1.28^{bB}	*0.001463*
		0.970638		*0.003979*	*0.002111*	*p value*
Lactobacillales						
amox02	2.97E+05 ± 2.75E+05^{A}	1.37E+09	9.26E+08 ± 4.18E+07	0.08^{A}	0.33^{A}	*0.064451*
amox07	2.53E+05 ± 1.67E+05^{A}	1.58E+09	9.26E+08 ± 4.18E+07	-0.10^{A}	-0.23^{A}	*0.085205*
amox08	5.33E+04 ± 2.89E+04^{B}	2.10E+09	9.26E+08 ± 4.18E+07 ^{a}	-2.59^{bB}	-2.96^{bB}	*0.000001*
blank	n.a.	1.58E+09	9.26E+08 ± 4.18E+07	-0.42^{A}	-0.34^{A}	*0.060350*
	0.043189	*0.999926*		*0.000002*	*0.000007*	*p value*
Bacteroides – Prevotella -Porphyromonas						
amox02	n.a.	5.91E+08	5.69E+08 ± 3.23E+07	-0.38^{A}	-0.68^{A}	*0.060603*
amox07	n.a.	5.42E+08	5.69E+08 ± 3.23E+07^{a}	-1.26^{bA}	-1.47^{bB}	*0.000001*
amox08	n.a.	6.02E+08	5.69E+08 ± 3.23E+07^{a}	-2.27^{bB}	-3.19^{cC}	*0.000002*
blank	n.a.	5.42E+08	5.69E+08 ± 3.23E+07^{a}	-0.41^{aA}	-0.81^{bA}	*0.000187*
		0.901999		*0.000615*	*0.000569*	*p value*
Bifidobacteriaceae						
amox02	1.24E+04 ± 2.67E+02^{B}	3.81E+02	2.35E+02 ± 1.42E+02	0.54	0.93^{A}	*0.712690*
amox07	2.34E+04 ± 3.63E+03^{A}	9.68E+01	2.35E+02 ± 1.42E+02	n.d.	n.d.	*n.d.*
amox08	n.d.	2.32E+02	2.35E+02 ± 1.42E+02^{a}	-4.37^{c}	-2.65^{bB}	*0.039056*
blank	n.a.	2.32E+02	2.35E+02 ± 1.42E+02	-0.15	-0.13^{A}	*0.946939*
	0.000001	*0.951484*		*0.088766*	*0.036499*	*p value*
Enterobacteriaceae						
amox02	1.80E+04 ± 5.36E+02^{B}	1.30E+07	1.39E+07 ± 1.31E+06	0.68^{A}	1.28^{AB}	*0.301428*
amox07	2.07E+04 ± 5.42E+03^{AB}	1.25E+07	1.39E+07 ± 1.31E+06^{b}	0.96^{aA}	2.56^{aA}	*0.000256*
amox08	3.50E+03 ± 2.39E+03^{A}	1.51E+07	1.39E+07 ± 1.31E+06^{a}	-1.42^{bB}	-2.54^{bC}	*0.003090*
blank	n.a.	1.50E+07	1.39E+07 ± 1.31E+06	0.12^{A}	0.35^{B}	*0.073044*
	0.000001	*0.907242*		*0.001772*	*0.000737*	*p value*
Clostridium group IV						
amox02	5.07E+04 ± 9.35E+03	2.51E+08	2.51E+08 ± 1.01E+07	-0.55	-0.57^{A}	0.074610
amox07	1.22E+04 ± 3.68E+03	2.45E+08	2.51E+08 ± 1.01E+07	-0.27	-0.30^{A}	0.436952
amox08	1.50E+04 ± 1.17E+04	2.64E+08	2.51E+08 ± 1.01E+07^{a}	-1.43^{b}	-3.02^{cB}	0.000072
blank	n.a.	2.44E+08	2.51E+08 ± 1.01E+07	-0.42	-0.27^{A}	0.080431
	0.307957	*0.993674*		*0.079424*	*0.000007*	*p value*
Clostridium group I						
amox02	2.07E+04 ± 6.10E+03^{A}	8.02E+06	1.07E+06 ± 5.67E+06	1.05^{A}	1.31^{A}	*0.003502*

amox07	3.42E+04 ± 3.15E+03^{A}	7.03E+06	1.07E+06 ± 5.67E+06	1.21^{A}	1.43^{A}	*0.004502*
amox08	2.49E+03 ± 1.16E+03^{B}	1.73E+07	1.07E+06 ± 5.67E+06	-1.30^{B}	-2.32^{C}	*0.046666*
blank	n.a.	1.20E+07	1.07E+06 ± 5.67E+06	-0.20^{A}	-0.28^{B}	*0.705529*
	0.000071	*0.987058*		*0.000275*	*0.000003*	*p value*
Escherichia coli						
amox02	2.03E+02 ± 5.33E+01^{A}	1.50E+06	1.63E+06 ± 1.36E+05^{b}	0.51^{abA}	1.48^{aA}	*0.000010*
amox07	1.33E+02 ± 2.64E+01^{B}	1.58E+06	1.63E+06 ± 1.36E+05^{b}	1.10^{aA}	1.07^{aA}	*0.041208*
amox08	n.d.	1.76E+06	1.63E+06 ± 1.36E+05^{a}	-1.49^{bB}	-3.50^{cB}	*0.000004*
blank	n.a.	1.66E+06	1.63E+06 ± 1.36E+05	0.15^{A}	0.38^{A}	*0.064937*
	0.000002	*0.933157*		*0.000001*	*0.000244*	*p value*

^{a,b,c} = Different lowercase letters on the superscript of values indicate significance difference due to "time category" of MANOVA among a row by Tukey post hoc test ($p < 0.05$). ^{A,B,C} = Different uppercase letters on the superscript of values indicate significance difference due to "substrate category" of MANOVA among a column by Tukey post hoc test ($p < 0.05$); n.a. = not analysed; n.d. = not determined (values under the threshold of 1.0E+2 cells/mL or over 36th cycle of amplification from the raw data of qPCR). BL = Baseline of colonic fermentation; SD = Standard Deviation; T1 = 18 h; EP = 24 h.

12.3.3. Metabolomics

12.3.3.1. Discrimination of the volatilome of different samples

Through SPME GC-MS, among 18 duplicated cases (n = 36), 158 molecules were identified with more than 80% of similarity with NIST 11 MSMS library and the NIST MS Search program 2.0 (NIST, Gaithersburg, MD, USA). On average, 96 were relatively quantified at the BL, while 137 were quantified during the 24 h of experiments at different timepoints. For a landscape description of the volatilome, a dataset of 56 significant molecules (ANOVA $p < 0.05$) was generated, then sorted and super-normalized by similar chemical classes of VOCs, i.e., aldehydes, alcohols, acids and ketones, and other aromatics (alkanes were excluded) (Nissen et al., 2020). In details, within the 17 aldehydes quantified, 6 were found at the BL, 16, 17, and 16, were found during fermentation of amox02, amox07, and amox08, respectively. Within the 14 alcohols quantified, 9 were found at the BL, 13, 14, and 12, were found during fermentation of amox02, amox07, and amox08, respectively. Within the 6 organic acids quantified, 3 were found at the BL, 5, 5, and 3, were found during fermentation of amox02, amox07, and amox08, respectively. Within the 6 ketones quantified, 4 were found at the BL, 6, 5, and 4, were found during fermentation of amox02, amox07, and amox08, respectively. From each dataset, multivariate analyses, such as untargeted Principal Component Analysis (PCA) (Figure 1) and targeted MANOVA ($p < 0.01$) (Table S3 and Table S4) were achieved to address the specific contributes to VOCs production by the independent variables. Super-normalization of the dataset was essential to unveil the effect of those compounds that are less volatile than others and could be underrepresented, as well as to avoid comparing one chemical class to another.

The results that we have presented have highlighted that in respect to the BL there were no fermentation differences for the blank control, but there were discriminations in respect to the milk fermentations, and that each one had typical descriptors mainly produced at the EP. This means that the fermentations of milk substrates produced different profiles of VOCs, because made a different impact on the colon microbiota.

A PCA of 14 statistically significant alcohols has distributed cases on the plot, discriminating the BL (M11 BL) variables to the fermentation of any milk samples, but not to that of the blank control (Fig. 1A). From our results, the main descriptors of fermentation with the milk samples were Butanol for amox02, 1-Heptanol and 2-Nonen-1-ol (E) for amox07, and 1-Nonen-3-ol and 2-Cyclohexen-1-ol for amox08 (MANOVA $p < 0.01$). Among alcohols the production of Butanol described the milk with no antibiotics, while 2-Cyclohexen-1-ol that of amox08. The colon microbiota produces different alcohols during fermentation of dietary polysaccharides. For example, Butanol is a product of butanoic acid fermentation that happens when the pH is not low enough to ensure the exclusive activity of lactic acid bacteria, as should happen in a healthy piglet colon, maybe due to the action of Clostridia. In fact, it is reported that *C. acetobutylicum* produces less acids and more neutral products like butanol, thus carrying out acetone butanol fermentation (Ciani et al., 2008). 2-Cyclohexen-1-ol was probably produced from microbial transformation of amoxicillin building blocks, like Cyclohexenone (Jiang et al., 2020). Considering the effect of time on the production of these VOCs, the major contributions were derived from the EPs (MANOVA $p < 0.01$).

A PCA of 16 statistically significant aldehydes showed distributed cases on the plot, separating the BL from the fermentations of milk samples, but not from the blank control (Fig. 1B). From our results, the main descriptors of fermentation with the milk samples were 2-Butenal, 2-methyl and Butanal, 3-methyl for amox02, Butanal for amox07, and Hexadecanal and 2-Nonenal, (Z) for amox08 (MANOVA $p < 0.01$). Benzeneacetaldheyde and 2-Butenal, 2-methyl were found to be descriptors of fermentation of milks with and without antibiotic residues, respectively. The aldehydes that are a result of microbial fermentation of lipids could be health-promoters, like 2-Butenal, 2-methyl that was reported to limit the growth of several intestinal pathogens at a very low concentration (Zhang et al., 2020) and could have contributed to the management of a natural occurring eubiosis of colon microbiota. Also other aldehydes are detrimental, being cytotoxic at a low threshold, such as Benzeneacetaldheyde (Zhang et al., 2020), that in our work could have been derived from bacterial fermentation of phenylalanine, that is typically rich in milk proteins. The higher amount of this

aldehyde found after fermentation of milk with antibiotic residues could have been produced by the higher abundance of *E. faecalis* that characterized the end of amox08 fermentation. In fact, this taxon is known for its selectivity in fermentation of phenylalanine in respect to lactobacilli (Canon et al., 2021). Considering the effect of time on the production of these VOCs, the major contribution for 2-Nonenal, (Z) was derived from T1 (18 h) and for the others the major contribution was derived from the EPs. (MANOVA $p < 0.01$).

A PCA of 12 statistically significant ketones and organic acids distributed cases on the plot, separating the substrates from each other and from the BL, except for the blank control (Fig. 1C). Descriptors of fermentation were Butanoic, Propanoic acids, and 2-Octanone for amox02, Pentanoic and Hexanoic acids for amox07, and Acetic acid for amox08.

A PCA of 13 statistically significant aromatic VOCs distributed cases on the plot, separating the substrates from each other and from the BL, but not form the blank control (Fig. 1D). Otherwise, considering the MANOVA, the main descriptors of fermentation were mainly addressed to amox08 cases. In particular, principal descriptors of this sample fermentation were Indole and Phenol.
So far, the volatilome of colonic fermentation of mother's milk containing antibiotic residues was described by positive features, such as higher acetic acid, but also by negative ones, such as the higher Indole and Phenol loads.

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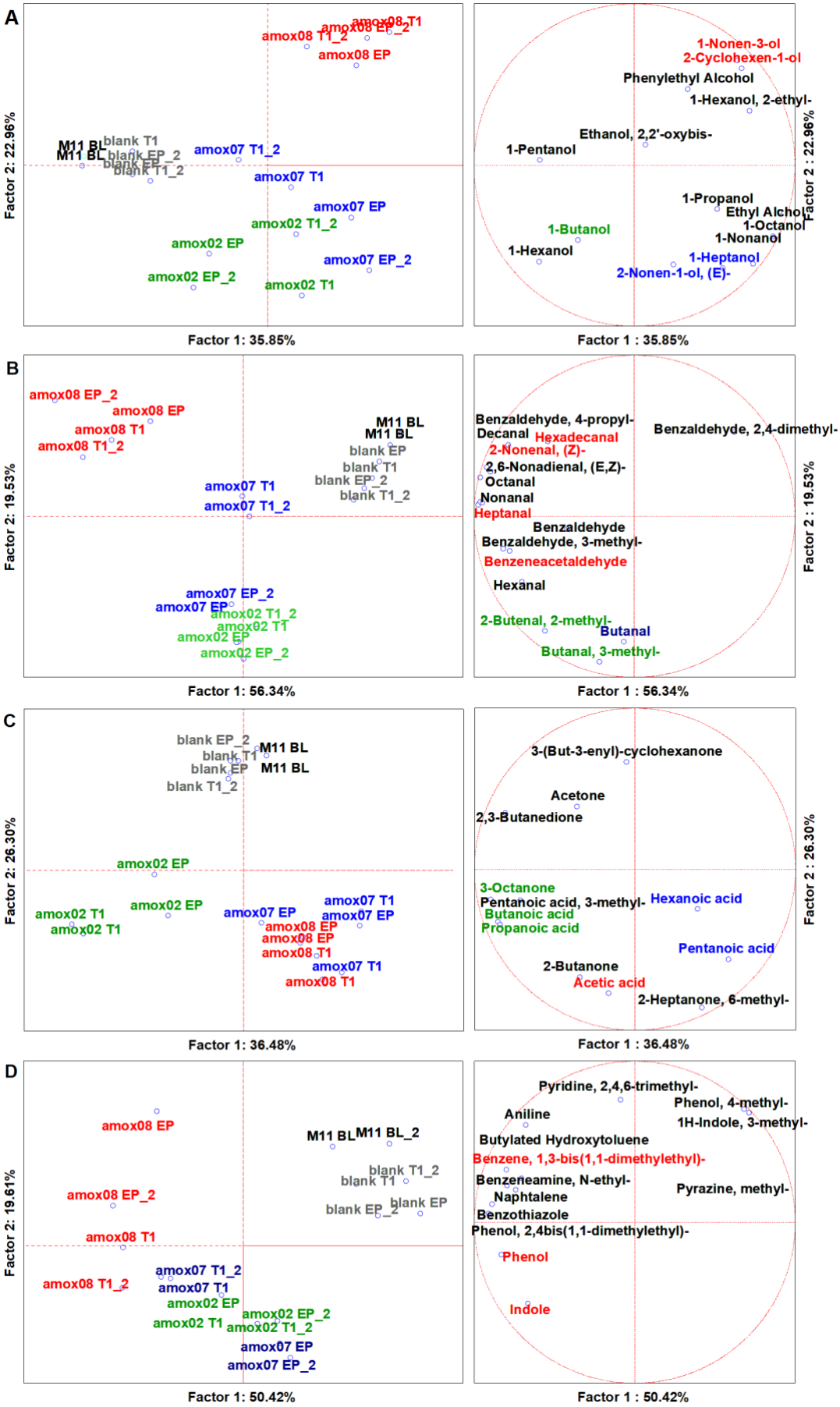

Figure 1. PCA of the volatilome of colonic fermentation samples. A = Alcohols; B = Aldehydes; C = organic acids and ketones; D = aromatic compounds. M11 BL = Baseline (2.10 h); T1 = Intermediate time point (18 h); EP = Endpoint (24 h). Different colors on variables indicates respective descriptors by MANOVA ($p < 0.05$) (Table S3 and Table S4).

12.3.3.2. Shift of beneficial or detrimental microbial metabolic indicators

To analyze the production of principal volatile microbial metabolites related to food fermentations, we have considered the quantity differences from the BL to the EP, including T1 of eight selected VOCs (ANOVA $p < 0.05$) with renowned bioactivity in humans (short and medium chain organic acids and aromatic compounds). In this elaboration of results we chose not to include the case of the blank control, because the output generated by volatilome analyses found no discrimination for this case. The first group of VOCs is relative to low organic acids, such as Acetic, Propanoic, and Butanoic acid, that are beneficial compounds essential for the piglets' gut mucosa and the eubiosis of the colon microbiota (Figure 2A). The second set is relative to compounds related to proteolytic fermentation and/or detrimental for the piglet's gut mucosa, such as Indole, 1H-Indole, 3-methyl (skatole), Phenol, Phenol, 4-methyl (p-cresol), and Benzeneacetaldehyde (Figure 2B).

Results shown in Fig. 2A indicate that Acetic, Propanoic, and Butanoic acids concentration was increased from small amounts detected at the BL (Table S2), with any milk sample. Specifically amox02 fermentation produced the top amounts of Propanoic and Butanoic acid, but little quantity of Acetic acid. In contrast the amox08 fermentation produced the top amount of Acetic acid, but little quantity of Propanoic and Butanoic acid. A reduction in Acetic, Propanoic, and Butanoic acids abundances is linked to dysbiosis of the colon microbiota and a reduced intestinal cell homeostasis (Gibson et al., 2017). Thus, from our results no sample was able to disrupt the proper colonic fermentation of milk, because the three of them increased the production of these VOCs in respect to the BL. The different scenario observed in the production of low organic acids could be principally addressed to the increased abundance of enterococci to the reduction of lactobacilli for the production of acetic acid, and to the reduction of butyrate-producers bacteria (e.g. *Ruminococcaceae* and *Lachnospiraceae*) seen in amox08.

Enterococci have pyruvate dissimilation that follows several pathways leading to at least five fermentation end-products including acetate (Snoep et al., 1991). In line with our results, Fujita et al., reported that the supplementation of pigs fed with *E. faecium* increased fecal acetate levels, which plays an important role for maintaining immune functions (Fujita et al., 2020). Oppositely, the reduced microbial production of butanoic acid seen in amox08 in respect to amox02 has to be linked to the undesired inhibitory effect of the antibiotic residues towards sensible commensal *Clostridiales*

that are butyrate producers. In particular, we have observed a reduction in (*Roseburia* and *Faecalibacterium prausnitzii*). Butanoic acid in piglets is produced mainly by the colon microbiota and is a preferential nutrient for energy production by the colonocyte (Kien et al., 2002). Also, it is an important modulator of gut cellular homeostasis, and when it is administered in diet as sodium butyrate alleviates diarrhea symptoms and decreased intestinal permeability without affecting the growth of early weaned piglets (Feng et al., 2018).

Results shown in Figure 2B indicate that starting from baseline values (Table S2) detrimental aromatic VOCs concentration trended similarly during any milk sample fermentation. Skatole and p-cresol were reduced, while Indole, Phenol, and Benzeneacetaldehyde were increased, after colonic fermentation in respect to the BL. In particular, there were significative differences between amox02 and amox08 in the production of Indole and Benzeneacetaldehyde and in the reduction of p-cresol. In particular, the former two were produced 3.9-folds more and 2.8 -fold more in amox08 than amox02, respectively. In pigs, Skatole and Indole are formed by the microbial degradation of L-tryptophan in the large intestine and contribute to the typical development of boar taint (Witte et al., 2021). L-tryptophan accumulates especially in the colon when protein sources are used with a low precaecal digestibility (Leong et al., 2011). The reduction of these compounds is due to the liver, but when their concentrations is excessive can accumulate also in the adipose tissue (Witte et al., 2021), resulting in a commercial loss. From our results the higher increase in indole of amox08 in respect to amox02 could be due to the reduced abundance of *Lactobacillaceae* observed in the presence of antibiotic residues. In fact, this taxon in the colon can transform Indole into beneficial compounds (*e.g.* Indole propionic acid) (Konopelski and Mogilnicka, 2022).

Similarly to indoles, Phenol and p-cresol are derived from proteolytic fermentation of undigested or partially digested proteins and have been shown to damage the gut mucosa disrupting the epithelial barrier function and being genotoxic (Al Hinai et al., 2019; Wang et al., 2020). Also in farm animals, the excessive production of these metabolites can affect the quality of meat and milk and is a source of contaminating emissions from animal manure (Gasaly and Gotteland, 2021). In our work, Phenol and p-cresol should be derived from fermentation of tyrosine due to proteolysis of milk. From our results, the capacity of amox08 fermentation to reduce less the amount of p-cresol than what the control milk did, could still be attributed to a lower content in *Lactoabacillaceae*, as it has been reported in a similar *in vitro* colon model, where the correlation among lactobacilli and p-cresol was negative (Al Hinai et al., 2019).

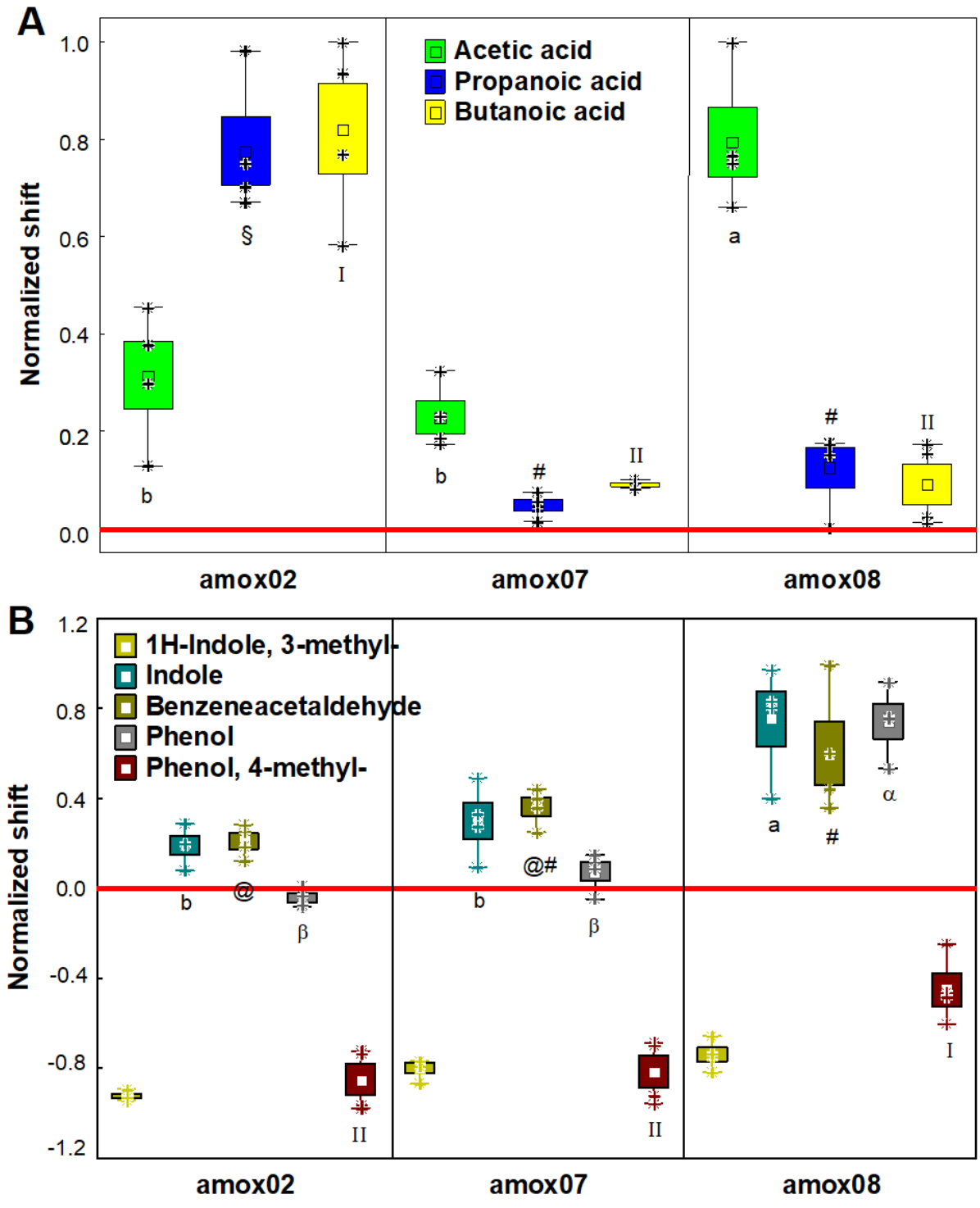

Figure 2. Changes in the abundance of (A) beneficial microbial VOCs metabolites and of (B) detrimental VOCs metabolites, expressed as normalized scale from relative abundances with respect to the baseline (red line). The baseline absolute quantifications in mg/kg are found in the Supplementary Material (Table S3). Changes were recorded after 18, and 24 h of *in vitro* fecal batch fermentations with amox02, amox07, amox08, and a blank control. Each plot is made with the raw data obtained from each time point and replica. Samples were analyzed in duplicate from two independent experiments (n = 4). Marker = mean; box = mean ± S.D.; whiskers = min-max; asterisks

= raw data. Cases with different letters or numbers or symbols among a single independent variable are significantly different according to Tukey's HSD test ($p < 0.05$).

12.3.4. Swine model

Within antibiotics for use in animals, The European Medicines Agency (EMA, 2020) has currently classed amoxicillin, without beta-lactamase inhibitors, as Category D antibiotic; therefore, it is highly recommended as first line treatment and, as always, should be used prudently only when medically needed (EMA/CVMP/CHMP/682198/2017). The establishment of the intestinal microbiota is a pivotal step in newborn piglets, thus the effects of antibiotics such amoxicillin in early life stages could critically affect gut microbial development and future growth (Lin et al., 2018; Mu et al., 2017; Yu et al., 2018). This statement is especially true amid zootechnical industry, where intensive farming pigs undergo fast and massive weight increment. The present *in vitro* experiment using an innovative colon model allowed authors to carry out a preliminary study avoiding any unnecessary harm to the piglets and the sows as well, still obtaining reliable data on microbial shift due to amoxicillin residues in sows' milk.

Digestive enzyme secretory patterns seem to be of relevance in the process of assimilation of milk components; indeed, previous studies showed that maturation of gastric, pancreatic and biliary digestive fluids occurs at an early period of life, starting gradual maturation around the sixth day of life (Corring et al., 1978; Harada et al., 1988; Manners, 1976). To the authors' knowledge, there are yet no researches that evaluated amoxicillin digestion and absorption in newborn piglets. In this study were used 7 days old piglets' fecal samples to build up the *in vitro* colon microbiota model; therefore, as the newborn had an immature digestive capacity, the milk samples were directly fermented in the colon model with no gastric phase digestion.

12.4. Conclusions

Since the early establishment of a stable gut microbiota is pivotal for the pigs' gastrointestinal physiological functions, also affecting future growth performance, investigating exogenous molecules effects on these indigenous microbes is of great importance in swine production. In this work pig model was adopted to study the role of sow's milk in modifying antibiotic resistant gut microbiota for the first time in combination to a gut model. Moreover, a wider understanding was allowed by a metabolomic approach. The use of MICODE, a robust and versatile *in vitro* model, together with multivariate statistics visibly demonstrated a suitable approach to describe the effects generated by milk containing amoxicillin residues towards the colon microbiota of suckling piglets. To fully understand the transfer of antibiotic from sow's milk to the piglets, *in vivo* trials are

imperative; however, the results presented are target-effective and should be reliable for pre-clinical investigations. Due to the results obtained, this experimental approach looks suitable to study some mechanisms of antibiotic resistance transfer as well. Furthermore, such *in vitro* approach could be included in a pipeline of experiments reducing the number of living animals testing, according to the Directive 2010/63/EU and the Regulation (EU) 2019/1010.

12.5. **References**

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12.6. Supplementary materials

Table S1. Primers pairs employed for PCR and qPCR reactions and quantifications.

Bacterial taxa	**Target**	**Sequence 3'-5'**	**Bp***	**MT** (°C)**	**Reference**
Eubacteria	*V3-V4 16S*	Eub518-R: ATTACCGCGGCTGCTGG Eub338-F: ACTCCTACGGGAGGCAG	147	57.6 63.5	Lane et al, 1991
Firmicutes	*V3-V4 16S*	Firm-F: GGAGYATGTGGTTTAATT Firm-R: ACTCCTACGGGAGGCAG	300	60.5 63.5	Guo et al, 2008
Bacteroidetes	*V3-V4 16S*	Bact-F: GGARCATGTGGTTTAATT Bact-R: AGCTGACGACAACCATG	250	58.9 59.4	Guo et al, 2008
Lactobacillales	*V3-V4 16S*	F-Lac: GCAGCAGTAGGGAATCT R-Lac: GCATTYCACCGCTACACA	340	59.8 58.3	Walter et al, 2001
Bifidobacteriaceae	*RecA*	RecA-F: CGTYTCBCAGCCGGAYA RecA-R: CCARVGCRCCGGTCATC	220	60.3 59.2	Masco et al, 2006
Enterobacteriaceae	*V3-V4 16S*	Enterb-F: TGCCGTAACTTCGGGAG Enterb-R: TCAAGGACCAGTGTTCAG	450	64.2 60.3	Bartosh et al, 2004
Clostridium group I	*V3-V4 16S*	ClosI-F: TACCHRAGGAGGAAGCCAC ClosI-R: GTTCTTCCTAATCTCTACGCAT	148	54.6 53.0	Bartosh et al, 2004
Clostridium group IV	*V3-V4 16S*	ClosIV-F: TTAACACAATAAGTWATC ClosIV-R: ACCTTCCTCCGTTTTGTC	400	58.1 57.9	Goldberg et al, 2013
BPP group	*V3-V4 16S*	BPP-F: GAGAGGAAGGTCCCCCA BPP-R: CGCKACTTGGCTGGTTCA	140	60.5 59.9	Pachikian et al., 2011
Escherichia coli	*FtsZ*	EcFtsZ-F: GGTATCCTGACCGTTGCT EcFtsZ-R: ATACCTCGGCCCAGAACT	250	59.4 57.3	Zhou & Helmstetter, 1994

*Base pairs, **Melting temperature.

Table S2. Quantification of VOCs by SPME GC/MS related to prebiotic potential, employing 10000 mg/kg of 2-Pentanol, 4-methyl.

VOCs	**mg/kg ± S.D.**	***p* value**†
Acetic acid	0.031 ± 0.010	0.0013
Propanoic acid	0.019 ± 0.012	0.0469
Butanoic acid	0.061 ± 0.021	0.0231
Indole	25.352 ± 9.795	<0.0001
Phenol	2.912 ± 0.594	<0.0001

Phenol, 4-methyl-	6.193 ± 1.231	0.0156
Benzeneacetaldehyde	0.020 ± 0.007	0.0212
1H-Indole, 3-methyl	0.843 ± 0.043	0.0096

*traces < 0.01 mg/kg; **Phenol, 2,4-bis(1,1-dimethylethyl)-; (LOQ = 0.03 mg/kg and LOD = 0.01 mg/kg); † *p* value of ANOVA from a dataset including all cases and time points.

Figure S1. Alpha and Beta Biodiversity of colon microbiota after 24 h of fermentation

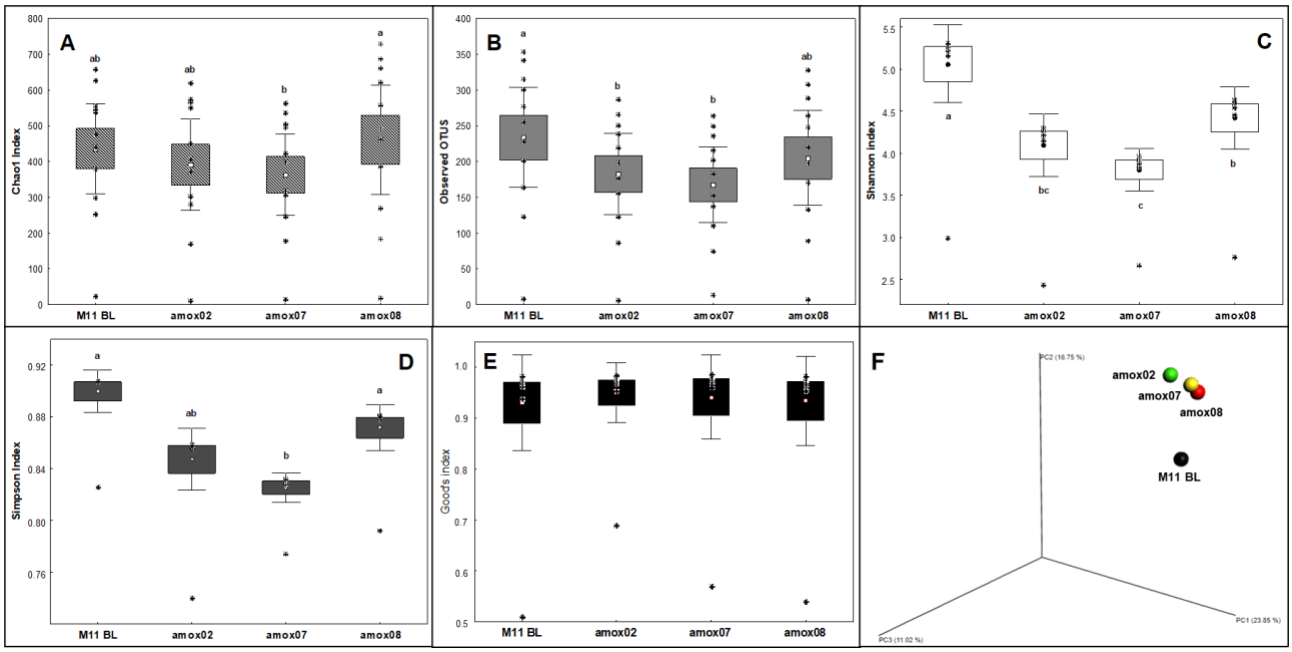

Table S3. Multivariate analyses of the volatilome of different samples at different time points during in vitro colonic fermentation, categorized by "Effect of Time" by MANOVA ($p < 0.05$).

VOCs	**Effect of time to dependent variables production (%)**			**MANOVA**
	BL	T1	EP	*p* Value
1-Butanol	37.48	23.73	38.78	0.766684
1-Heptanol	2.49	34.82	62.68	0.410634
1-Hexanol	63.35[a]	18.98[b]	17.66[b]	0.019447
1-Hexanol, 2-ethyl-	0.01	47.40	52.59	0.524728
1-Nonanol	0.01	47.41	52.58	0.089240
1-Nonen-3-ol	0.01	46.82	53.17	0.555965
1-Octanol	0.01[b]	40.04[a]	59.95[a]	0.021290
1-Pentanol	90.12[a]	0.023[b]	9.84[b]	0.000000
1-Propanol	32.73	27.75	39.50	0.477265
2-Nonen-1-ol, (E)-	0.01	72.52	27.47	0.110141
2-Cyclohexen-1-ol	0.01	48.95	51.04	0.145041
Ethyl Alchol	5.91[b]	36.11[ab]	57.97[a]	0.038833
Ethanol, 2,2'-oxybis-	14.62[b]	68.73[a]	16.64[b]	0.027514

Phenylethyl Alcohol	24.53	24.87	50.58	0.573784
Butanal	0.01	20.23	79.76	0.102151
Butanal, 3-methyl-	0.01	48.17	51.82	0.306084
2-Butenal, 2-methyl-	0.01	49.08	50.91	0.115503
Hexanal	6.90	41.97	51.11	0.147786
Heptanal	0.01	46.17	53.82	0.153683
Octanal	0.01	45.34	54.65	0.126945
Nonanal	0.01	45.60	54.39	0.141468
2-Nonenal, (Z)-	0.01	41.07	58.92	0.311281
Decanal	0.01	48.24	51.75	0.263056
2,6-Nonadienal, (E,Z)-	0.01	50.03	49.96	0.248240
Hexadecanal	0.01	56.94	43.05	0.376285
Benzaldehyde	22.50	42.38	35.10	0.659388
Benzaldehyde, 2,4-dimethyl-	51.20	25.18	23.61	0.084450
Benzaldehyde, 3-methyl-	0.01	59.64	40.35	0.066304
Benzaldehyde, 4-propyl-	27.37	34.12	38.49	0.909027
Benzeneacetaldehyde	2.05	57.81	40.13	0.123854
Benzene, 1,3-bis(dim*)-	26.79	38.14	35.05	0.787677
Benzeneamine, N-ethyl-	32.01	42.15	25.82	0.033509
Benzothiazole	26.58	43.51	29.90	0.153335
Butylated Hydroxytoluene	32.02^{ab}	41.08^{a}	26.88^{b}	0.032881
1H-Indole, 3-methyl-	61.81^{a}	20.46^{b}	17.72^{b}	0.029024
Indole	13.95	39.87	46.17	0.224445
Naphtalene	29.10	41.11	29.78	0.378391
Phenol	37.03	24.88	38.07	0.649405
Phenol, 2,4bis(dim*)-	23.74	43.29	32.96	0.299933
Phenol, 4-methyl-	65.96^{a}	18.33^{b}	15.69^{b}	0.036611
Aniline	34.39	40.30	25.30	0.393777
Pyrazine, methyl-	0.01	47.77	52.22	0.310295
Pyridine, 2,4,6-trimethyl-	42.34	26.54	31.11	0.318745

*1,1-dimethylethyl

Table S4. Multivariate analyses of the volatilome of different samples at different time points during in vitro colonic fermentation, categorized by "Effect of Substrates" by MANOVA ($p < 0.05$).

VOCs	**Effect of substrates to dependent variables production (%)**				**MANOVA**
	Blank Control	amox02	amox07	amox08	*p* Value
1-Butanol	28.56^{b}	52.87^{a}	9.75^{c}	8.81^{c}	0.037014
1-Heptanol	1.67	30.32	58.25	9.75	0.207862
1-Hexanol	53.54^{a}	28.41^{b}	18.05^{b}	0.00^{c}	0.000187
1-Hexanol, 2-ethyl-	0.01	18.65	22.32	59.03	0.232363

CASE STUDY 8*:* Amoxicillin treatment

1-Nonanol	0.00[c]	37.77[a]	42.60[a]	19.62[b]	0.033886
1-Nonen-3-ol	0.00[c]	0.00[c]	30.11[b]	69.89[a]	0.020889
1-Octanol	0.00	33.75	40.51	25.73	0.074134
1-Pentanol	85.88[a]	0.38[c]	7.02[b]	6.71[b]	0.000003
1-Propanol	24.49	22.96	28.96	23.57	0.916492
2-Nonen-1-ol, (E)-	0.00[b]	38.30[a]	61.69[a]	0.00[b]	0.006394
2-Cyclohexen-1-ol	0.00[c]	8.67[b]	36.52[a]	54.80[a]	0.000001
Ethyl Alchol	4.02	27.27	38.75	29.95	0.193970
Ethanol, 2,2'-oxybis-	10.25	24.15	31.01	34.58	0.800229
Phenylethyl Alcohol	17.81	18.41	8.11	55.66	0.135020
Butanal	0.00[b]	42.43[a]	57.56[a]	0.00[b]	0.042168
Butanal, 3-methyl-	0.00[c]	62.24[a]	24.25[b]	13.50[b]	0.000000
2-Butenal, 2-methyl-	0.00[c]	45.84[a]	28.00[b]	26.14[b]	0.000205
Hexanal	4.08[b]	36.62[a]	26.04[a]	33.24[a]	0.030241
Heptanal	0.00[c]	23.48[b]	23.90[b]	52.60[a]	0.000003
Octanal	0.00[c]	23.70[b]	24.57[b]	51.71[a]	0.000000
Nonanal	0.00[c]	24.31[b]	23.68[b]	52.00[a]	0.000003
2-Nonenal, (Z)-	0.00[c]	18.76[b]	16.30[b]	64.93[a]	0.000133
Decanal	0.00[c]	13.88[b]	27.19[b]	58.92[a]	0.000000
2,6-Nonadienal, (E,Z)-	0.00[c]	18.56[b]	22.68[b]	58.75[a]	0.000000
Hexadecanal	0.00[c]	0.00[c]	39.14[b]	60.85[a]	0.000004
Benzaldehyde	15.08	21.95	28.77	34.18	0.699660
Benzaldehyde, 2,4-dimethyl-	45.53	18.81	13.74	21.90	0.002611
Benzaldehyde, 3-methyl-	0.00[c]	30.27[b]	26.72[b]	43.03[a]	0.004375
Benzaldehyde, 4-propyl-	19.15	13.73	19.31	47.79	0.198589
Benzeneacetaldehyde	1.17[c]	29.66[ab]	26.36[b]	42.79[a]	0.027948
2,3-Butanedione	35.63[b]	64.35[a]	0.00[c]	0.00[c]	0.000000
2-Butanone	9.90[b]	34.35[a]	31.76[a]	23.97[a]	0.016575
2-Heptanone, 6-methyl-	0.00[c]	17.42[b]	44.23[a]	38.34[a]	0.000001
3-(But-3-enyl)-cyclohexanone	41.89[a]	19.37[b]	24.83[ab]	13.89[b]	0.038087
3-Octanone	0.00[c]	96.08[a]	3.91[b]	0.00[c]	0.003803
Acetone	32.06	26.84	29.65	11.43	0.077502
Acetic acid	0.00[c]	35.34[a]	16.58[b]	48.06[a]	0.001296
Butanoic acid	0.00[c]	86.61[a]	7.02[b]	6.35[b]	0.017478
Propanoic acid	4.52[c]	72.47[a]	0.00[d]	23.00[b]	0.037235
Pentanoic acid, 3-methyl-	12.49[b]	74.33[a]	13.16[b]	0.00[c]	0.012310
Pentanoic acid	0.00[c]	2.48[c]	66.17[a]	31.33[b]	0.000515
Hexanoic acid	2.48	2.62	84.58	10.31	0.127988
Benzene, 1,3-bis(dim*)-	14.78	37.59	22.68	24.93	0.167975
Benzeneamine, N-ethyl-	19.02	26.05	22.84	32.08	0.450401
Benzothiazole	14.62[b]	27.01[ab]	21.83[ab]	36.53[a]	0.019382
Butylated Hydroxytoluene	19.02	29.07	22.26	29.63	0.349089
1H-Indole, 3-methyl-	65.35[a]	10.07[b]	9.87[b]	14.69[b]	0.000000
Indole	6.54[c]	26.17[b]	26.76[b]	40.51[a]	0.000001

CASE STUDY 8*:* Amoxicillin treatment

Naphtalene	16.57	24.28	30.45	28.68	0.520621
Phenol	23.75[b]	7.04[c]	17.38[bc]	51.81[a]	0.035000
Phenol, 2,4-bis(dim*)-	12.57	30.87	26.62	29.92	0.086084
Phenol, 4-methyl-	78.26[a]	8.24[b]	6.41[b]	7.07[b]	0.000000
Aniline	21.16	33.95	17.97	26.89	0.739773
Pyrazine, methyl-	0.00[b]	36.96[a]	34.82[a]	28.20[a]	0.014902
Pyridine, 2,4,6-trimethyl-	29.65[a]	25.78[ab]	13.04[b]	31.52[a]	0.049508

*1,1-dimethylethyl-

13. CONCLUSIONS

In this thesis we assessed a new colon model (MICODE) working under a strict control of ecosystem parameters (**Chapter 3**). The fermentation process set up and monitoring was performed by pc in remote. We also assessed an output panel for data interpretation based on microbiomics and metabolomics indicators, scientifically robust and suitable to carry out clear and repeatable results. Beneficial or detrimental indicators were tested in all the following experiments.

Therefore, we tested MICODE model on eight case studies representative for the most common food substitutions/additions/fortifications.

In **CASE STUDY 1**, bread fortified by polyphenols rich olive fiber (PFR) resulted with an increased prebiotic potential attributable to PRF addition based on the favorable results obtained by several prebiotics indicators (**Chapter 4**). Moreover, SCFA and MCFA production increased as well as a decline in the abundance of dangerous BCFAs like Indole and Skatole. Additionally, in fortified bread, there was a drop in opportunistic or pathogenic species and an increase in probiotic or helpful organisms. A combined information from microbial genomes and metabolomics provided an interomic display that clearly illustrates the causes and consequences brought on by a specific fiber with prebiotic potential.

In **CASE STUDY 2** we compared a sourdough standard gluten free (GF) bread to a sourdough fortified GF bread with *Arhtrospira platensis* powder (AP) to enrich the protein content (**Chapter 5**). We concluded that GF sourdough containing AP could mitigate the negative effect of GF standard sourdough in a healthy ecological condition of the human colon. In this context, the GF sourdough with AP in comparison to the standard one is able to; i) preserve microbial eubiosis (more *Bacteroidetes*); ii) increase the abundance of beneficial bacterial groups, such as *Bifidobacterium*, *Akkermansia, Roseburia, and Faecalibacterium* and *Ruminococcus* from *Ruminococcaceae*; iii) limit opportunistics, sulfurate producers, and proteolytic bacteria, such as *Escherichia*, *Bilophila*, and *Clostridium* from *Clostridiaceae*); iv) produce more bioactive low organic fatty acids; v) reduce detrimental compounds, such as p-cresol; vi) generate a prebiotic effect. Otherwise, we have to consider that an exacerbation of the proteobacteria loads was observed during any fermentation, suggesting that there are few beneficial impact of GF breads on a healthy colon.

In **CASE STUDY 3**, innovative formulations were evaluated in which the nitrites were replaced by ascorbic acid and / or a mix of plant antioxidants (**Chapter 6**).
Results showed that the innovative formulations promote a general eubiosis of the intestinal microbiota, in the face of those preselected indices including favorable F / B ratio, proliferation of beneficial microbial taxa including *Lactobacillales, Bifidobacteriaceae* and reduction of negative microbial populations, including *Enterobacteriaceae* and ATOP group. Furthermore, the volatiloma analysis highlights a marked production of beneficial molecules, including short-chain fatty acids such as Acetate, Propionate and Butyrate, and a reduction in host negative molecules such as Phenol and p-Cresol, resulting from the fermentation of proteins. Although the nitrites substitution did not provide benefits clearly superior to those of the controls, the results obtained are promising, as the antioxidants used in place have given results comparable to those obtained with the traditional formulation.

In **CASE STUDY 4** where a fiber-based ingredient (FLS) with limonene was considered (**Chapter 7**), it was possible to unveil the prebiotic potential of FLS which was similar to the prebiotic capacity exerted by FOS but delayed (probably because of limonene). Indeed, the EO terpenes and terpenoids present in FLS probably played an antimicrobial activity at the early and intermediate time points of fermentations implicating a slower production of beneficial or reduction of detrimental compounds. From a fast microbial turnover and high production of beneficial compounds is foreseen as a good characteristic of a prebiotic, but the capacity to slow microbial metabolism as well as to contribute to a more stable microbial yield and composition over time could be useful for those consumers that are more susceptible to physiological imbalances. After *in vivo* studies this supplement is now in the marketplace as limenorm® (https://www.tgd.care/it/prodotti/limenorm/).

In **CASE STUDY 5** in **Chapter 8** we considered the prebiotic potential of hempseed bran. Basing on the positive results obtained by different beneficial or harmful indicators, our study evidenced that HB (hempseed bran) and in particular HBPA (alcalase-treated HB protein isolate) had a prebiotic potential comparable to that of FOS. Furthermore, the populations of beneficial and fiber degrading bacteria were fostered and in contrast those of opportunistic and proteolytic bacteria were limited by HBPA and minorly by HB colonic fermentations. Additionally, alcalase treatment of HB increased prebiotic activity probably due to a higher release of substrates for the fermentation by beneficial microbes, and nasty or even toxic for the fermentation by opportunistic microbes.

In **CASE STUDY 6 (Chapter 9)** we report for the first time the effects of lactose-free milk, compared to the control containing lactose, on a batch colonic fermentation model simulating human colonic microbiota from healthy and lactose-intolerant subjects.

The results showed that the fermentation of lactose resulted in an effective insult for the HCM of lactose-intolerant adults, resulted by the depletion of commensals butyrate producers (*Ruminococcaceae* and *Lachnospiraceae*), and commensal fibrolytic *Bacteroidaceae* and by the raise in dysbiotic and diarrhea inducers, either at the phylum and family levels (*Proteobacteria* and *Enterobacteriaceae*) and also by the raise of opportunistic *Peptostreptococcaceae.* Considering, the changes in the metabolites production during colonic fermentation, we evidenced the negative effect of lactose presence towards the HCM of lactose intolerant adults, as the reduction in production of Butanoic acid, possibly linked to the depletion of butyrate-producers' taxa.

On the contrary, the absence of lactose generated an insult for the host HCM of healthy adults, demonstrated by the depletion of the beneficial populations of the microbiota, *Lactobacillales* and *Bifidobacteriaceae* and, considering the changes in the detrimental metabolites production during the fermentation, by the production of Indole.

In CASE STUDY 7 (Chapter 10) we focused on lactose-free dairy products to investigate the effect of lactose free fermented whey on the intestinal microbiota of both healthy subjects and lactose-intolerant individuals.

The results showed that the fermented whey SPF has a positive effect on the gut flora of healthy subjects, increasing the production of beneficial metabolites (e.g. SCFA) and reducing the amount of potentially toxic metabolites (e.g. Phenol and p-Cresol). However, the results related to the microbiota showed a greater positive effect with not fermented whey SP, favouring the proliferation of beneficial microbial taxa, including *Bifidobacteriaceae,* and the reduction of negative microbial populations, including potentially toxic *E. coli,* although without significant differences compared to the other samples.

In the case of lactose intolerant subjects, however, in terms of volatile analysis, the same positive result was obtained with SP, but promising results were also obtained with SPF. Although their viability was very low, the addition of probiotics to fermented whey was found to be more desirable for lactose intolerant people.

A quite different application was the use MICODE as pig model in **CASE STUDY 8** to explore the role of sow's milk in modifying antibiotic resistant gut microbiota that combined together for the first time pig model to an in vitro gut model (**Chapter 12**). Moreover, a wider understanding was allowed

by a microbiomic and metabolomic approach to describe the effects generated by milk containing amoxicillin residues towards the colon microbiota of suckling piglets. Due to the results obtained, this experimental approach looks suitable to study some mechanisms of antibiotic resistance transfer as well. Furthermore, such combination of in vivo and *in vitro* models could be included in a pipeline of experiments reducing the number of living animals testing, according to the Directive 2010/63/EU and the Regulation (EU) 2019/1010

14. FINAL REMARKS AND FUTURE PERSPECTIVES

In a recent survey for EURL ECVAM (Batista Leite et al. 2021) on stakeholders it has been established a renewed interest in some kind of assessment of experimental approaches for the more complex in vitro models. Indeed, testing food, feed, supplements, and drugs in rodents (and other animal species) for human health and safety purposes has been questioned repeatedly. In addition to the ethical issues that arise from this, the transferability of animal data across species is often problematic because of differences in physiology, metabolism and chemical susceptibilities.

To reduce the number of experiments (and thereby cost and time), one potential way is to first simulate and build models of such an ecosystem and suggest top ranking models for experimental validation. In this regard, gut microbial community has been mimicked, although under certain controlled conditions, using *in-vitro* fermentation models suitable to identify mechanisms that lead to beneficial or detrimental effect on human health. Although the first studies in gut models started in 1988 (Gibson et al., 1988) there is a lack of standardization in studies based on colon models while it was recently done for gastro-duodenal ones (Infogest method).

This work demonstrated that the colon model MICODE here purposed resulted as a reliable and adaptable *in vitro* model, which was evaluated by a quality control check of various issues, including the presence of *Archea* species throughout the fermentation period, the ability of FOS to foster probiotics, the of similar observed OTUs in the system, as well as the rare species seen by Good's index, and, lastly, taking into account the volatilome, there were several stool-derived compounds kept at the same retention time. Even if *in vivo* animal models or diet-intervention studies should be used to fully elucidate the prebiotic potential of foods as well as to address specific host benefits, the recipient results we have presented are target-effective and robust enough for pre-clinical applications. That makes MICODE together with its flexibility one of the potential candidates to study a standardization of experimental methods finalized to a consensus protocol for *in vitro* colonic fermentations.

14.1. References

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CHAPTER 14: Final remarks and future perspectives

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