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"Characterization of novel probiotics and prebiotics"

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"Since we cannot know all that there is to be known about anything,
we ought to know a little about everything"

Blaise Pascal

"We are unconscious of most of our body's processes, thank goodness,
because we'd screw it up if we weren't.
The human body is so complex, with so many parts...
The idea that we are consciously caretaking
a large and mysterious system is ludicrous."

Lynn Margulis

Abstract

The role of the human gut microbiota in impacting host's health has been widely studied in the last decade. Notably, it has been recently demonstrated that diet and nutritional status are among the most important modifiable determinants of human health, through a plethora of presumptive mechanisms among which microbiota-mediated processes are thought to have a relevant role.

At present, probiotics and prebiotics represent a useful dietary approach for influencing the composition and activity of the human gut microbial community.

The present study is composed of two main sections, aimed at elucidating the probiotic potential of the yeast strain *K. marxianus* B0399, as well as the promising putative prebiotic activity ascribable to four different flours, naturally enriched in dietary fibres content.

Here, by *in vitro* studies we demonstrated that *K. marxianus* B0399 possesses a number of beneficial and strain-specific properties desirable for a microorganism considered for application as a probiotics. Successively, we investigated the impact of a novel probiotic yoghurt containing *B. animalis* subsp. *lactis* Bb12 and *K. marxianus* B0399 on the gut microbiota of a cohort of subjects suffering from IBS and enrolled in a *in vivo* clinical study. We demonstrated that beneficial effects described for the probiotic yoghurt were not associated to significant modifications of the human intestinal microbiota.

Additionally, using a colonic model system we investigated the impact of different flours (wholegrain rye and wheat, chickpeas and lentils 50:50, and barley milled grains) on the intestinal microbiota composition and metabolomic output, combining molecular and cellular analysis with a NMR metabolomics approach. We demonstrated that each tested flour showed peculiar and positive modulations of the intestinal microbiota composition and its small molecule metabolome, thus supporting the utilisation of these ingredients in the development of a variety of potentially prebiotic food products aimed at improving human health.

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Human gut microbiota: what is it?

Human beings are colonised by several microbial communities, which have the potential to impact on the host's health. Different human districts, such as skin, mouth, vagina, airways and gastrointestinal tract (GIT), harbour specific bacterial ecosystems. Anatomically, the human GIT is divided into six sections, the oral cavity, oesophagus, stomach, small intestine (duodenum, jejunum and ileum), the colon or distal gut (ascending, transverse and descending colons) and rectum (Fig. 1). Each GIT section is characterised by a specific microbial community, which to some extent reflects the physiology and the dynamics of that compartment. The number of microbes in each niche increases as one moves from the stomach to the rectum resulting in one of the most densely populated ecosystems being found in the distal gut or colon. Indeed, the most rich and complex microbial consortium resides in the colon, reaching a bacterial concentration of 100-200 billions of cells/gram of faeces, so that the number of microbial inhabitants within the gut lumen can achieve 10^{14} (Marchesi et al., 2010; Marchesi et al., 2011; van den Bogert et al., 2011). Even if the human intestinal microbiota is one of the most densely populated microbial ecosystems in nature, sequence-based analysis demonstrated that it is characterized by a peculiarly low phylogenetic diversity (Fig. 2).

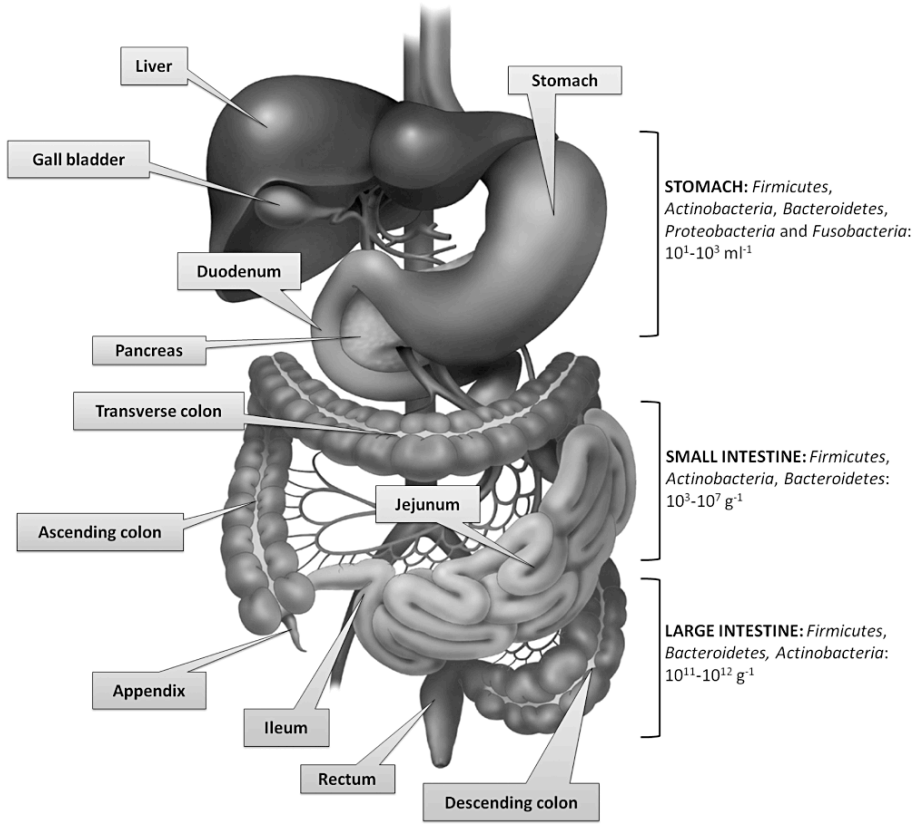


Figure 1. The anatomy of the gastrointestinal tract, major bacterial phyla and their abundance in each niche. (from Marchesi et al., 2011)

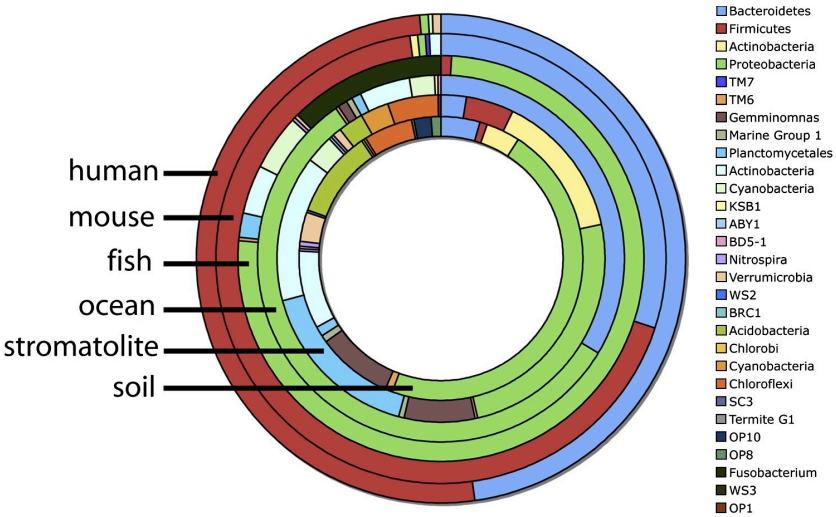


Figure 2. Comparison of microbial diversity in the human colon against other deeply studied animal and environmental ecosystems. Percent representation of divisions in each environment is reported by Ley and colleagues (2006).

Out of the over 70 phyla described to date, Next Generation Sequencing (NGS)-based survey indicated that 6 to 10 is the number of bacterial phyla per individual represented in the gut microbiota: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*, candidate division TM7, *Verrucomicrobia* and *Deniococcus-Thermus* (Marchesi et al., 2010; 2011). Among these, *Firmicutes* and *Bacteroidetes* represent up to 90% of the intestinal microbiota, with a relative abundance of approximately 65% and 25%, respectively (Fig. 3).

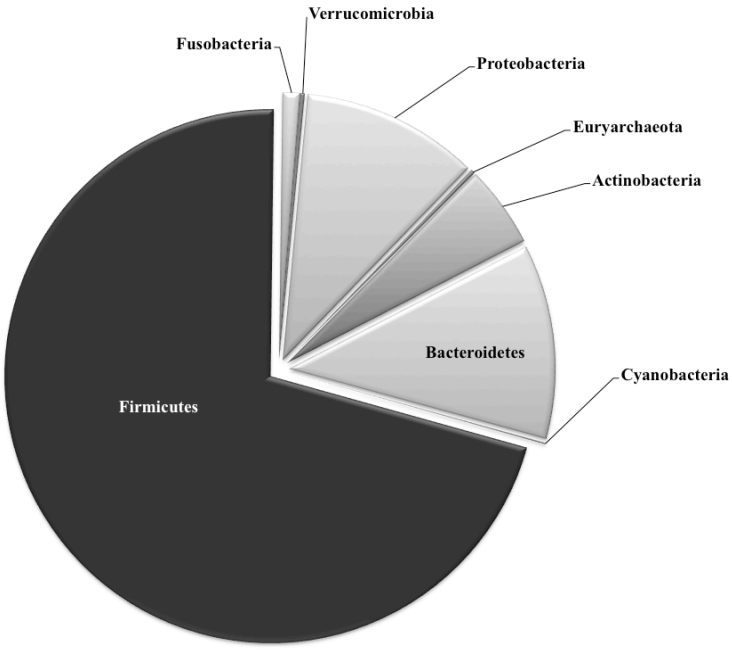


Figure 3. Relative proportion of the phylotypes belonging to the 8 main bacterial divisions which have been found in the human gut microbiota, out of the over 70 globally known (Candela et al., 2010).

A great diversity at lower taxonomic levels and a considerable inter-individual variability in the bacterial species and strains have been described. In fact, the number of species have been estimated to range in the hundreds (Qin et al., 2010) and a bloom at phylotype level have been reported, with about 16,000 phylotypes estimated to thrive in the human intestine (Peterson et al., 2008). A consequence of this phylotypes diversity is that phylogenetic trees of the gut tent to have few branches, which are not deep, but have a large degree of radiance at the ends (Fig. 4). Interestingly, 70% of these phylotypes are subject-specific, none of which is present at more than about 0.5% abundance in all subjects. Furthermore, metagenomics studies

demonstrated that about 65% bacteria identified in the intestinal microbiota were previously unknown, and among them 80% were uncultivable (Turnbaugh et al., 2009).

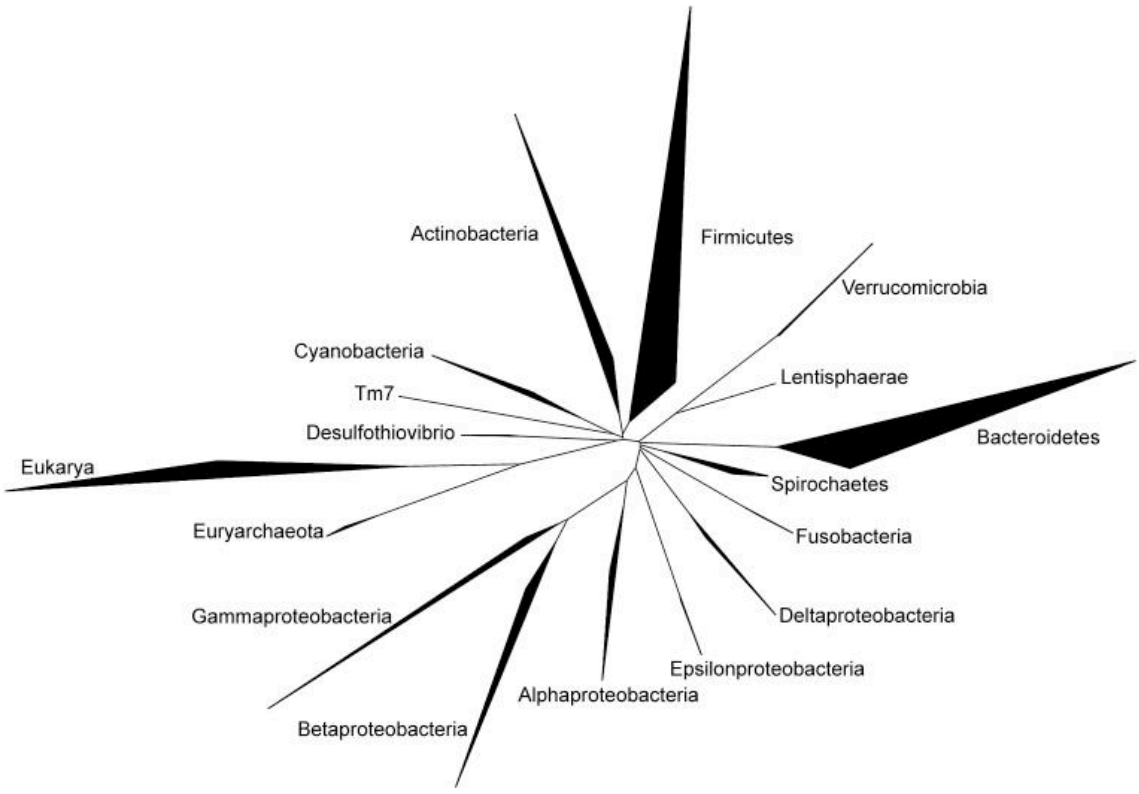


Figure 4. Phylogenetic tree representing the groups of bacteria most frequently detected in human faeces using 16S rRNA gene sequencing. The extent of the bold areas indicate diversity and abundance of the bacterial groups. The phylogenetic tree is reported by Vrieze and colleagues (2010).

While studies looking to define the core microbiota have focused on describing the Bacteria within the human gut microbiota, a significant number of Archaea is populating this ecological niche. The most common species found in the distal gut come from the *Euryarchaeota* and in particular the *Methanobacteriaceae* family (Scanlan et al., 2008; Dridi et al., 2009) with *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* the two predominant inhabitants. However, other rarer archaeal sequences that cluster in the *Methanosarcinales* (Scanlan et al., 2008), *Halobacteriaceae* (Oxley et al., 2010) and a putative sixth archaeal order (Mihajlovski et al., 2008; 2010) have been reported.

Nevertheless, in all studies to date *M. smithii* and *M. stadtmanae* are the two main *Archaea* (Dridi et al., 2011), whilst it is still unclear to what extent the much rarer species are autochthonous or are actually contaminants from our diet/environment (Marchesi et al., 2011).

The gut microbiota additionally contains subdominant micro-eukaryotic and viral components that can interact with symbiotic bacteria to play a primary role in both ecosystem structure and function. Most of the researches undertaken on the eukaryotic members of the gut ecosystem have been culture-based approaches targeting fungal diversity (*Candida* and *Saccharomyces* spp.). Only recently culture-independent approaches looking at the micro-eukaryotic fraction of the human gut microbiota have been applied (Scanlan and Marchesi, 2008). The micro-eukaryotic diversity and concentration are several orders of magnitude lower than the bacterial ones. 18S rRNA gene-based methods have been used, demonstrating that the genus *Blastocystis* and *Ascomycota* were the predominant micro-eukaryotes populating the intestinal ecosystem (Scanlan and Marchesi, 2008), whilst yeast are rarely obtained. Indeed, it has been proposed that the micro-eukaryotic fraction of the intestinal microbiota might have a significant role only in presence of a dysbiosis (Goldman and Huffnagle, 2009). Dysbiosis has not only been associated to yeast and micro-eukaryotic cells, but Lepage and colleagues (2008) have hypothesized a role for distal gut bacteriophages as driver of dysbiosis in the distal gut leading to IBD. Very recently, Reyes et al. (2010) used a NGS approach to study the virome, the collective metagenome of virus-like particles isolated from human faecal samples. They demonstrated that 81% of reads generated in the metagenomics study did not match to any known viruses, and that the human virome consists of prophages or phages generally classified as temperate (i.e., coliphage P22-like), commonly hosted by *Firmicutes* and *Bacteroidetes* members.

Why a human intestinal microbiota?

With ≥100 times as many genes as our 2.85 billion base pair human genome, the collective genome of our symbiotic intestinal microbiome endows humans with crucial physiological traits that have not been evolved in his own (Gill et al., 2006; Neish, 2009; O'Hara and Shanahan, 2006; Xu et al., 2007). These traits are mainly belonging to three functional macro-categories: protective, structural and metabolic functions (Fig. 5).

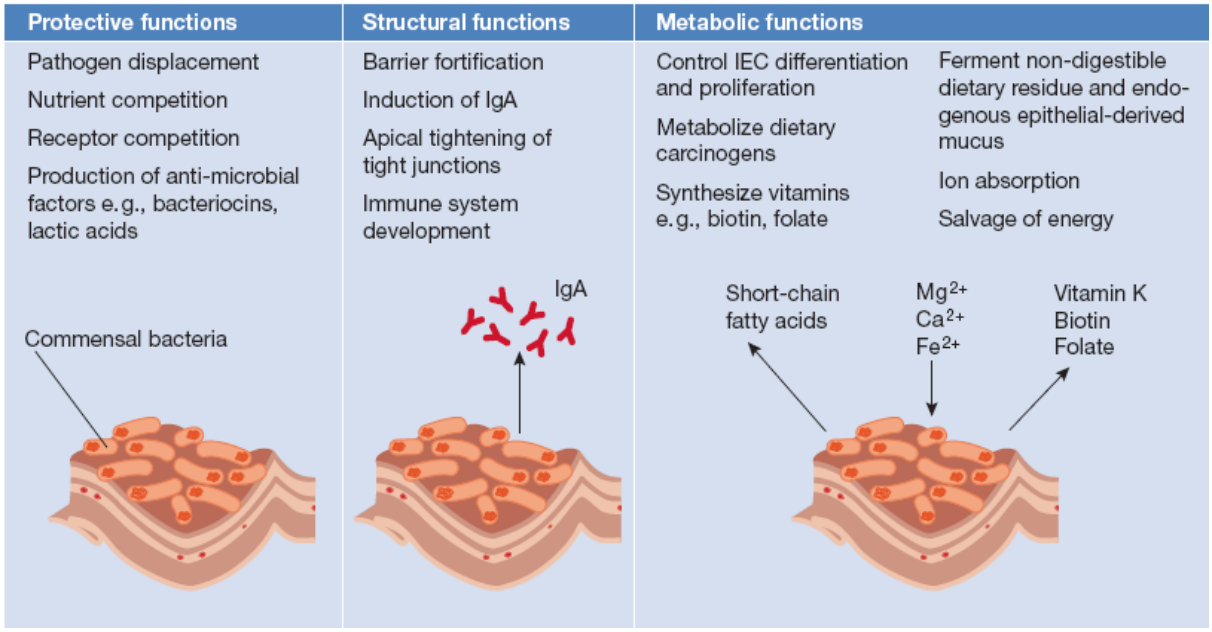


Figure 5. The human intestinal microbiota exert several protective, structural and metabolic functions on the intestinal mucosa (from O'Hara and Ferguson, 2006).

The human intestinal microbiota exerts a pivotal contribution to the human energy balance and nutrition. Throughout the extension of the host metabolic capacity to indigestible polysaccharides, the microbiota has the intrinsic ability to produce short chain fatty acids (SCFAs), which constitute a fundamental energy source for human colonic epithelium and provide from 5 to 15% of the total energy requirement (Neish, 2009; Box n. 1). The intestinal microbiota can further affect the absorption of key mineral and can synthesize several vitamins involved in different host metabolic pathways, as cobalamin (vitamin B_{12}), pyridoxal

phosphate (the active form of vitamin B_6), pantothenic acid (vitamin B_5), niacin (vitamin B_3), biotin, tetrahydrofolate and vitamin K (Kau et al., 2011).

BOX N. 1 - THE SCFAS PARADIGM

The SCFAs paradigm represents a relevant example of how nutrient processing by the human intestinal microbiota and host diet combine to shape immune responses. SCFAs are the main end products of the microbial fermentations of plant polysaccharides that cannot be digested by humans, since our genomes do not encode the large repertoire of glycoside hydrolases and polysaccharides lyases needed to cleave the varied glycosidic linkages present in the dietary glycans, i.e host glycans enclosed in mucus and glycans from plant polysaccharides (xylan-, pectin- and arabinose-containing carbohydrate structures) (Qin et al., 2010). Indeed, the human intestinal microbiota is endowed with a real arsenal of carbohydrate-active enzymes (CAZymes), many of which are not present in the human glycobiome. The percentage of sequences in the gut microbiome assigned to CAZymes is greater than all the other KEGG pathways, indicating the abundance and diversity of microbiome genes directed towards the metabolism of a wide range of polysaccharides (Candela et al., 2010).

In addition to acting as an energy source for the host, SCFAs exert notable effects on host immune responses. Levels of butyrate modify the cytokine production profile of immune competent cells and promote intestinal epithelial barrier integrity, thus leading to preventing aberrant inflammatory responses (Jacobs et al., 2009; Kau et al., 2011). Recent studies highlighted the importance of the SCFA acetate in preventing infection by the enteropathogenic *Escherichia coli* (O157:H7) (Fukuda et al., 2011), as well as the possibility that SCFAs may regulate the acetylation of lysine residues, a covalent modification which can have a role in innate and adaptive immune responses (Kim et al., 2010).

Intestinal microorganisms exert a strong impact also on energy storage by interacting with the host lipoprotein lipase (LPL)-mediated process for triglyceride storage in adipocytes. In particular, throughout the suppression of the intestinal epithelium expression of the LPL-inhibitor fasting-induced adipose factor (Fiaf), the intestinal microorganisms promote the

absorption of polysaccharides from the gut lumen (Bäckhed et al., 2004). Moreover, intestinal microorganisms are able to increase the glucose uptake in the host intestine and produce a substantial elevation in serum glucose and insulin, stimulating the hepatic lipogenesis (Delzenne et al., 2011).

The microbiota forms an integral part of the natural mechanisms of mucosal surfaces that prevent the organism from pathogenic microorganisms. In physiological conditions, it prevents attachment and multiplication of pathogenic or virulent microorganisms on the intestinal surfaces, as well as it plays a critical role in avoiding the invasion of these microorganisms into epithelial cells and the circulation (Tlaskalová-Hogenová et al., 2011).

Moreover, the fine and dynamic cross-talk between intestinal microorganisms and GIT immune system is crucial for its development and homeostasis (Sansonetti and Medzhitov, 2009). The host-microbe interface is characterized by the dual necessity to peacefully coexist with symbiotic microorganisms and to quickly respond to the microbial pathogens to which we are intermittently exposed. Studies carried out on germ-free mouse models revealed that the entire structural development of our GIT depends on the dynamic interaction with the intestinal microbiota (Sansonetti and Medzhitov, 2009). In fact, intestinal microorganisms educate the host immune system to tolerance against harmless antigens whilst, at the same time, concur in the maintenance of a fast responsiveness towards harmful pathogens. In this context, the human intestinal microbiota contributes to the development and function of the immune system and it has been demonstrated that germ-free animals have altered compositions of $CD4^+$ T cells and IgA-producing B cells in the lamina propria, as well as the induction of T-lymphocyte subset is augmented by distinctive species of the luminal microbiota (Ashida et al., 2011). In addition, intestinal microorganisms exert a role of primary importance in the proper structuring of inductive and effector sites of the host GIT immune system (Garrett et al., 2010).

Humans and intestinal microbiota: when and how?

Recent technological advancement in the phylogenetic analysis of the microbial communities inhabiting the human ecosystems provided a huge number of novel insights about the development of the intestinal microbiota during the ageing process (Fig. 6).

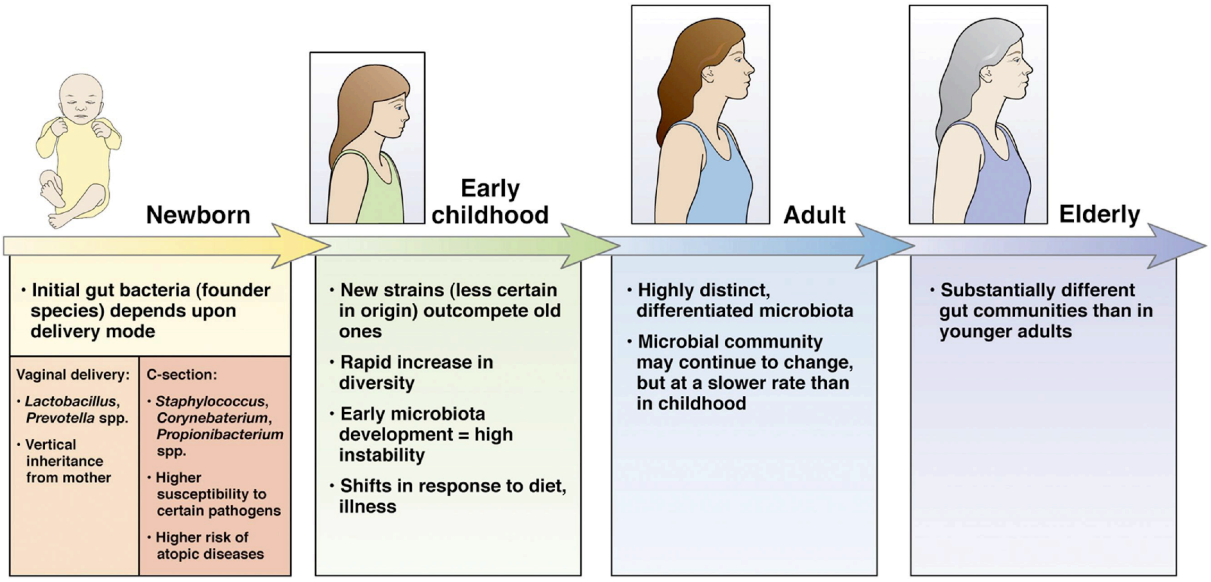


Figure 6. Diagram of the development of the microbiota from the first inoculum as an infant through continued change, modified by diet, genetics and the environment, throughout life (from Dominguez-Bello et al., 2011)

It is still questionable if newborns are sterile and are colonized after birth by environmental microbes or if the birth canal, which is heavily colonized by microbes, provide the primary inoculum for the baby. From an evolutionary perspective, it is unlikely to be accidental the relevant bacterial load in the birth canal, and it is thought that the vagina has likely evolved to provide the primary inoculum for the newborns (Dominguez-Bello et al., 2011). Indeed, recent community-wide metagenomics studies revealed that vaginally delivered babies acquire, at birth, their own mother's vaginal microbiota. Therefore, neonates' different body sites are colonized with essentially the same microbiota that was inherited vertically from their mothers, which is dominated by *Lactobacillus* and *Prevotella* spp. (Dominguez-Bello et

al., 2010). After the primary inoculation at birth, infants continue to have multiple exposures to human microbes and, only after a not yet well established time frame, babies develop the distinct microbial communities found in each body district in adulthood.

During the ageing process, humans are involved in a complex and dynamic interplay with environmental microbes that, after weaning, culminates in the acquisition of an adult-type intestinal microbial community (Palmer et al., 2007; Biagi et al., 2011). In particular, infancy is a crucial period of human life, since changes in diet and environment are reflected in relevant fluctuations in the GIT microbiota composition. This microbial plasticity providess efficient means for adaptation to the changing circumstances of development (Dominguez-Bello et al., 2011).

Advantages and limits of the classic approaches

Traditional culture-dependent techniques are based on selective culturing, morphological, biochemical, and physiological assays. This array of microbiological analyses requires laborious and time consuming cultivation of microorganisms, and allows the recovering of less than 20-30% of the total bacterial richness because of the insufficient anaerobic cultivation technologies, as well as the poor knowledge about the specific bacterial carbon source requirements (Bik et al., 2006). For this reason the vast majority of the biodiversity of the human microbiota remains uncultured, and the assessment of the microbial composition and abundance of such a dense and complex microbial community needs to be performed through molecular techniques. The mostly used molecular marker for genetic diversity of bacteria is the 16S rRNA gene, due to the fact that this gene is conserved in all eubacteria and present an appropriate balance of conservation and variability to allow discrimination between different species and strains, as well as identification and assignation to particular phylogenetic groups (Sekirov et al., 2010).

The 16S rRNA gene consists of about 1,500 nucleotides and contains regions conserved among all the bacteria, interspersed with 9 regions (V1 to V9, Fig. 7), which are highly variable among bacterial phylotypes (groups with 97-99% of sequence identity).

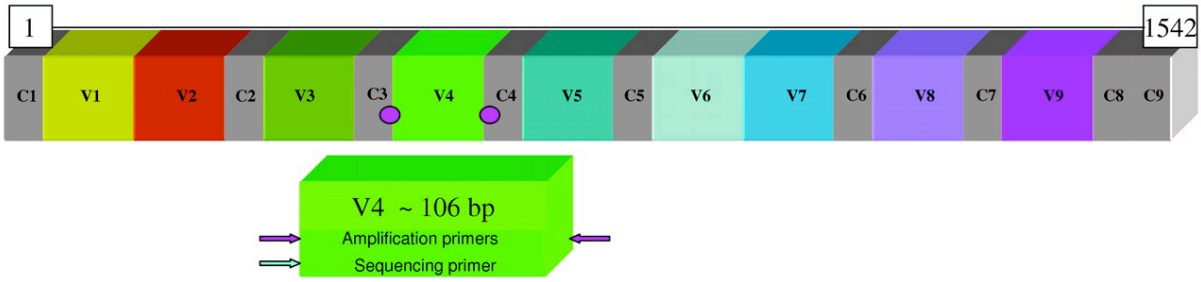


Figure 7. Conserved (C1-C9, grey) and hyper-variable (V1-V9, different colours) regions in the 16S rRNA gene, from Petrosino et al. (2009).

Conserved regions can be used as targets for PCR primers with almost-universal bacterial specificity. Phylotypes identification is obtained by comparative sequence analysis of the amplicons using available databases, as the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/). Different 16S rRNA-based techniques are available for the characterization of complex bacterial ecosystems (Box n. 2), and the choice of the approach depends on the question to be answered. By the use of fingerprinting techniques, as PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), an approximate "picture" of the most abundant bacterial components is obtained, but these approaches are usually not quantitative. Conversely, quantitative PCR (qPCR) and Fluorescent *In Situ* Hybridization (FISH), can quantify one or few bacterial groups targeted by specific primer sets or probes. Recently, several phylogenetic microarray platforms have been developed allowing simultaneous quantification of many bacterial components of the targeted ecosystem, even if this approach lacks the possibility to discover unknown members. In the last years, the phylogenetic characterization of the gut microbiota has greatly benefit from the combination of these approaches. However, none of them can give as accurate information as the most modern meta-omics approaches and next generation sequencing techniques.

BOX N. 2 - ADVANTAGES AND LIMITS OF THE MAIN MOLECULAR TECHNIQUES FOR THE GUT MICROBIOTA CHARACTERISATION

✳ **PCR-based DNA profiling techniques**. PCR-based DNA profiling techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal-restriction fragment length polymorphism (T-RFLP). The use of these techniques can provide a quick global assessment of the microbiota with a semi-quantitative measurement; however, these techniques are not quantitative for population size and are unable to provide detailed information on bacterial identities and phylogenic distribution.

✳ **Quantitative PCR (qPCR)**. qPCR is a commonly used method to perform absolute or relative quantification of specific bacterial group in the gut microbiota. qPCR assays are highly sensitive and provide accurate measurements. However, qPCR assays are normally limited to the measurement of one or few target bacterial species per assay under the conditions that the target gene sequence of known bacterial groups/species must be available in advance. Therefore, the main limitation of qPCR is that an individual assay is unable to provide a global assessment of bacterial communities.

✳ **Fluorescent in situ hybridization (FISH)**. Flourescein-labeled oligonucleotide probes targeting 16S rRNA are widely used for the gut microbiota characterization. Each probe has its own specificity to recognize a particular group of bacteria. Bacterial cells can be visualized by microscopy or flow cytometry. FISH is a quantitative method, which can provide information on the spatial distribution of target bacteria in the sample and can detect uncultured bacteria without enrichment. However, the sensitivity of FISH is relatively low. The sequence of target genes must be available in the database and only a few probes can be used at one time.

✳ **Phylogenetic DNA microarray**. A number of new DNA phylogenetic microarray have been reported in literature, based on 16S rRNA genes and Small-Subunit (SSU) rRNA genes. The advantages of microarray include high-throughput, cost-effectiveness, direct phylogenetic identification, and speed. However, detection limits, cross-hybridization and hybridization biases (i.e., some sequences hybridize more readily than others) are needed to be addressed and optimized when gut microbiota microarrays are used. Also, the targets sequences must be known in advance and hence it is not possible to identify novel species/strains of bacteria.

Omics science and the gut microbiota: "United, we stand"

The development of high throughput sequencing techniques and other affordable approaches allowing a large-scale analysis of microbial communities resulted in the application of 'omics' technologies, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics (Simon and Daniel, 2011). These techniques allow to analyze DNA, mRNA, proteins and metabolites of the gut microbiota and then unravel the complex diversity and functions within the gut microbial ecosystem.

Metagenomics refers to culture-independent and sequencing-based studies of the collective set of genomes of mixed microbial communities (metagenomes), and aims at exploring their compositional and functional characteristics (Gill et al., 2006; Petrosino et al., 2009). Using metagenomics, the analysis of large data sets allowed the exploration of the taxonomic and functional biodiversity and of the systems biology study of diverse ecosystems, among which the gut microbiome.

While in the last decade Sanger sequencing was used to generate data in most microbial genomics and metagenomics sequencing projects, NGS technologies have been widely used in the last 5 years to study the complex microbial ecosystem populating the GIT. In fact, community structures can now be investigated, bypassing previously needed cloning and cultivation procedures, at much lower cost than Sanger sequencing, and at much higher resolution by revealing rare or less abundant taxa.

NGS phylogenetic analysis of the gut microbiota is based on the amplification of selected target regions of the 16S rRNA genes. In particular, regions V1, V2, V3 and V6 have been used for studying the human GIT ecosystem. Two main platforms have been developed for NGS studies: i) Genome Sequencer 454 FLX system, that generally produces around 400,000 reads with average lengths of 250-350 bp, a read size sufficient to cover most of the variable regions in the 16S rRNA gene; ii) Illumina Genome Analyzer system, that routinely produces

more than ten times the number of reads per run as the 454 FLX system with average lengths of 35-75 bp.

To date, large-scale analyses of genomic and metagenomic sequences have provided gene catalogs and statistical evidence on protein families involved in the predominant functions of the human gut microbiome (Gill et al. 2006; Flint et al. 2008; Turnbaugh et al. 2009; Qin et al. 2010). In particular, functional metagenomics approach provides an experimental proof of function to highly prevalent genes and gene clusters of the human gut microbiome. Coupling sequence-based and activity-based metagenomics, the entire genetic and metabolic potential of the human microbiota are able to answer not only the "who's there" question, but also "what can they do together?".

Metatranscriptomics is based on high-throughput RNA sequencing and can be used to obtain functional insights into the gut microbiota as well as information about how environmental and host changes induce community-wide alterations in gene expression. RNA-sequencing (RNA-Seq) is a major technique used in metatranscriptomics analysis and its application to the human intestinal microbiota has been recently described (Gosalbes et al., 2011; Turnbaugh et al., 2010). The interest in human gut metatranscriptomics is relevant, because differently from metagenomic DNA-based analyses, which cannot differentiate between expressed and non-expressed genes, it provides data reflecting the actual metabolic activity of the ecosystem.

Metaproteomics, also referred to as whole community proteomics, is a new emerging function-based approach to identify key microbial functions in the community (Ram et al., 2005). Non-targeted mass spectrometry (MS)-based shotgun proteomics approach was recently used to detect and identify the array of proteins contained within the gut metaproteome without the need for gel-based separation or *de novo* sequencing (Verberkmoes et al., 2009). The advantages of metaproteomics are the direct monitoring of microbial protein

expression levels and the identification of new functional genes. To date, metaproteomics has been successfully used to analyze the complex proteome of the human distal gut microbiota (Verberkmoes et al., 2009; Zoetendal et al., 2008).

Metabolomics aims at quantifying all metabolites in a cellular system under defined conditions and at different time points so that the dynamics of any biotic, abiotic, or genetic perturbation can be accurately assessed. Metabolic profiling studies are mainly adopting MS and Nuclear Magnetic Resonance (NMR) spectroscopic platforms to measure the metabolome of biological samples. Indeed, the metabolic composition of fecal extracts provides a potent tool for elucidating the complex metabolic interplay between the symbiotic bacterial populations, as well as their interaction with the host (Holmes et al., 2011) or their response to nutritional intervention (Jacobs et al., 2009). In particular, metabolomics approaches that combine NMR profiling with multivariate pattern-recognition techniques are providing a holistic view of the perturbations of metabolism in response to diet or disease. NMR-based metabolite profiling is a well-established technique producing rapid, robust, and reproducible profiles without extensive sample preparation. It is a comprehensive but not very sensitive method (Jacobs et al., 2009). The metabolomics approach has been predominantly applied to urine, plasma and faecal extracts, and provides information on the exogenous and endogenous metabolism of the host.

In the next future, the challenge of the scientific community would be to integrate these complementary meta-omics data into an eco-systems biology approach to study the human microbiome.

Diet and nutritional status are among the most important modifiable determinants of human health. Recent studies emphasized the huge impact of nutrition, which is likely to outweigh that of the host genotype (Gophna et al., 2011). Diet composition has been demonstrated to provoke substantial effects on specific groups of bacteria constituting the human core microbiome (Walker et al., 2011). In particular, diet-driven modifications occur rapidly, being detectable within 1-4 days, and are reversed in a comparable time course (Kau et al., 2011; Walker et al., 2011). Assuming that the mean transit colonic time is ranging from 48 to 72h (Macfarlane et al., 1998), these kinetics appear to be consistent with immediate effects of dietary residue upon relative bacterial growth rates in the colon.

Very recently, a limited number of fascinating studies have been shedding light to the the host-diet-microbiota dynamic interplay. Walker et al. (2011) studied the diet-dependent microbiota dynamics in overweight men subjected to diets selectively enriched in main types of fermentable carbohydrates. A number of fluctuations in response to the different diet was demonstrated. Interestingly, the individual specificity of the gut microbiota was challenged by diet-driven changes in the major bacterial phylotypes.

Investigating the impact of long- and short-term dietary impact on the gut microbiota, Wu et al. (2011) found that higher fat intake and lower fiber intake are associated with particular bacterial groups. The Authors studied the influence of diet on the gut microbiota in the context of the enterotypes theory recently proposed by Arumugam et al. (2011; Box n. 3). Notably, enterotypes are determined by long-term dietary habits. Conversely, short-term controlled dietary intervention, whilst modulating rapid and significant modifications to the overall asset of our microbial complement within the first 24 h (Walker et al., 2011), did not provoked stable changes to the pattern of intestinal microbial populations sufficient to switch individuals from an enterotypes to another one.

BOX N. 3 - THE ENTEROTYPES OF THE HUMAN GUT MICROBIOME

In 2011, Arumagam et al. performed the first extensive work of annotation of predicted gene functions on 22 *de novo* Sanger-sequenced European gut metagenomes, combined with 17 existing Sanger and pyrosequencing gut data sets. These data were mapped to 1,511 reference bacterial genomes, including 379 publicly available human microbiome genomes generated by the Human Microbiome Project and the European MetaHIT consortium. The Authors were able to assign 53% of the sequenced fragments to a genus and the 80% to a phylum.

Notably, multidimensional cluster analysis and PCA revealed that samples formed three distinct clusters, that the Authors named “enterotypes”. Each of these three enterotypes are identifiable by the variation in the levels of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3). A functional analysis of the enterotype assets indicated that each enterotype use different routes to generate energy from fermentable substrates available in the colon. Enterotypes 1 and 2 were enriched in biosynthesis of vitamins, whereas enterotype 3 were enriched in membrane transporters, mostly for sugars.

It is noteworthy that enterotypes do not seem to differ in functional richness, and virtually host properties, i.e. nationality, gender, age or body mass index, significantly correlates with the enterotype. Conversely, functional properties, as host immune modulation and other physiological conditions might explain and allow classification of human groups into the three enterotypes.

Taken together, the current researches on the response of the intestinal microbiota to diet lead to a dynamic view in which the microbiota is continuously evolving in adaptation to long- and short-term dietary habits (Thomas et al., 2011). Diet may select peculiar differences in the intestinal microbiota that are strictly associated to geographical distribution. A comparative study between rural children in Burkina Faso (Africa) and Italian children subjected to Western lifestyle, showed that the intestinal microbiota of African children was enriched in *Bacteroidetes* and *Actinobacteria*, and depleted in *Firmicutes* and *Proteobacteria*. (De Filippo et al., 2010; Fig. 8) The Authors explained this difference by the higher dietary fiber content of the rural African diet, mainly composed of cereals, legumes and vegetables, which would favor the development of the polysaccharide-degrading *Bacteroidetes*, provided with an arsenal of carbohydrate-active enzymes (CAZymes) useful in acquiring energy from these undigestible sources. Furthermore, the types of *Bacteroidetes* present in the African children's microbiome differed from those in typical European microbiomes, as they may be ideally suited to grow on polysaccharides abundant in the Burkina Faso diet, such as xylan or cellulose.

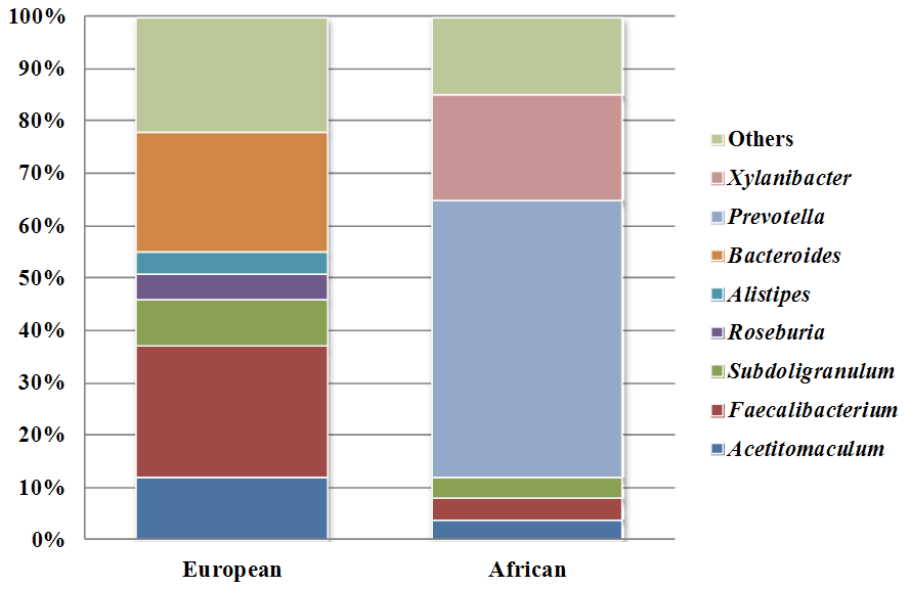


Figure 8. Differences between the intestinal microbiota of rural African children and European children, as reported by De Filippo et al. (2010)

It is noteworthy to understand how the selection of the microbial populations during the evolution resulted in a clear functional distinctions, conferring an advantage to those bacterial groups that optimally degrade the available substrates. As demonstrated by Muegge et al. (2011), gene encoding for enzymes involved in amino acid metabolisms are differentiating carnivorous and herbivorous. Microbiomes from herbivores are enriched in enzymes that map to biosynthetic reaction for a number of amino acids, whilst enzymes involved in the amino acid degradation pathways are significantly enriched in carnivores. These results suggest that diet played a crucial role also in the evolution, allowing microbiomes of carnivorous to specialize in the proteins degradation as an energy source, whereas microbiomes of herbivorous have specialized to synthesize amino acid building blocks. Recently, Walter and Ley (2011) reviewed the role of human evolution on changes to the intestinal microbiome. Notably, the invention of cooking has been described as a milestone for the evolutionary history of the microbiome and it represent a valuable paradigm in understanding how the gene content of the human microbiome have been shaped. Cooking induced the introduction of new toxins, i.e. the acrylamide produced by Maillard reaction, and other xenobiotics which can be further transformed by gut bacterial genes, as the beta-glucuronidases which appear to be unique to the human gut ecosystem (Gloux et al., 2011; Walter and Ley, 2011). Supporting the hypothesis that different human populations with different diets reflect specific food adaptation on the genetic level of their gut microbes, it has been recently demonstrated that acquisition of novel genes by the resident microbiota is a suitable method of adaptation. Hehemann et al. (2010) reported the first experimental evidence that the consumption of non-sterile foods containing environmental bacteria can lead to the acquisition of functional genes giving a metabolic advantage to the host. *Bacteroides plebeius*, a peculiar member of the microbiome from Japanese people, recently acquired the porphyrinase gene from the seaweed-associated marine bacterium *Zobellia galactanivorans* thanks to an event of lateral

gene transfer (LGT). The LGT of porphyrinase gene was a gut evolutionary response favored by the consumption of non-roasted dietary seaweed in Japanese sushi. Notably, this gene is undetected in North American microbiomes, and its presence in the microbiome allows Japanese to extract energy from the red marine alga porphyrin through bacterial fermentation of this indigestible polysaccharide to SCFAs in the gut.

That the intestinal microbial diversity of the human gut was the result of coevolution between microbial communities and their hosts was already proposed by Ley et al. (2006). In fact, it was suggested that the peculiar structure of microbial diversity in the human gut was the result of a natural selection operating at two levels: i) an host level, "top-down" selection on the community, favoring stable societies with a high degree of functional redundancy; ii) an opposing, "bottom-up" selection force, driving microbial cells to become functionally specialized (Fig. 9).

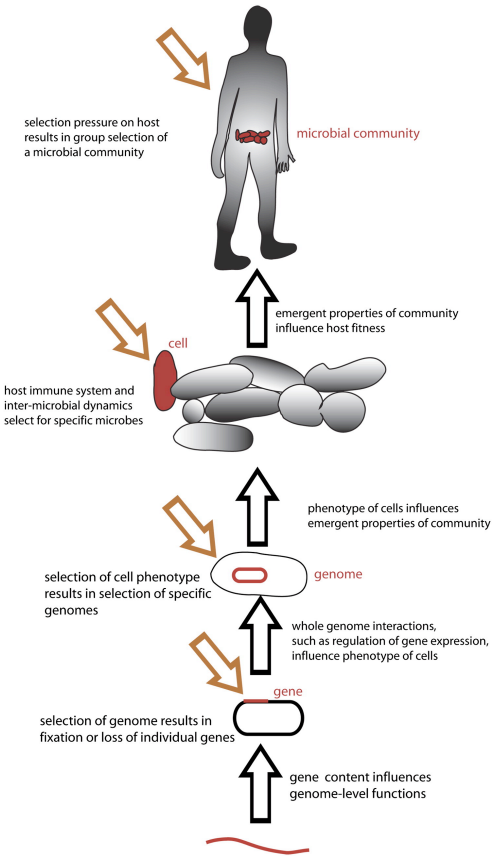


Figure 9. Schematic diagram of the selection processes operating in the human intestinal microbiota. Figure was reported by Ley et al. (2006); brown arrows indicate selection pressures, whereas black arrows indicate emergent properties acquired thanks to evolution.

Probiotics and human health

The Nobel Laureate Élie Metchnikoff is considered to be the inventor of probiotics. In 1907, Metchnikoff reported that "there are many useful microbes, amongst which the lactic bacilli", recommending "to absorb large quantities of (these) microbes". In particular, intrigued by the longevity of the Caucasian population and its frequent consumption of fermented milks, Metchnikoff proposed that the acid-producing microorganisms in fermented dairy products could prevent fouling in the large intestine and thus lead to a prolongation of the life span of the consumer (Metchnikoff, 1908).

According to the Food and Agricultural Organization of the United Nations and the World Health Organization, probiotics are "Live microorganisms, which, when consumed in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Notably, the definition does not mention the human origin of the bacterial strains as criteria for the selection and utilization of probiotics and, vice versa, it is based on the type of effect caused on the host. Accordingly, probiotics include exogenous and indigenous bacterial species that interact with various cellular components within the intestinal ecosystem.

To be considered as probiotics, microorganisms should fulfill the following criteria: i) being non-pathogenic and non-toxic; ii) being able to survive through the GIT; iii) being stable during the intended product shelf life and contain an adequate number of viable cells to confer health benefit to the host.

Nowadays, the consumer market for probiotic foods is > 1.4 billion euros with an estimated annual growth of ~7–8% in the 2008-2013 period (Saxelin, 2008) and in particular up to 20% of the fermented dairy products contains probiotics (Wassenaar and Klein, 2008). Marketed probiotic formulations commonly contain specific lactic acid bacteria strains, mainly belonging to *Bifidobacterium* or *Lactobacillus* genera. Conversely, less frequently used are strains of *Propionibacterium freudenreichii*, bacilli or yeasts (Wassenaar and Klein, 2008).

Many studies indicated probiotics as promising in the treatment of acute gastroenteritis, *Clostridium difficile*-associated and antibiotic-associated diarrhea or colitis, irritable bowel syndrome, allergy and maintenance of remission in inflammatory bowel diseases (Floch et al., 2011; Preidis and Versalovic, 2009).

Probiotics have been demonstrated to exert health promoting effects through several proposed mechanisms (Fig. 10), which rely on microbe-gut epithelium, microbe-immune system and microbe-microbe interactions. These mechanisms include: i) SCFAs production and enhancement of the barrier function of the intestinal epithelium; ii) suppression of growth and binding of pathogenic bacteria; iii) increased mucin production; iv) induction of antimicrobial and heat-shock protein production; v) alteration of the immune activity of the host through modulation of host signaling pathways (Aragon et al., 2010; Ventura et al., 2009; Thomas and Versalovic, 2011). Furthermore, probiotics can alter colonic fermentation and stabilize the symbiotic microbiota (Spiller, 2008), improving the dynamic interplay between the resident bacterial community and the host.

Since intestinal epithelial cells (IECs) are an initial point of contact between the host and intestinal microbes, adhesion to the intestinal epithelium is an important requisite for allowing probiotics to exert a beneficial role. Adhesion ability is strongly strain-dependent, therefore an evaluation of this characteristic is required as a selection criterium for novel probiotics (Collado et al., 2009).

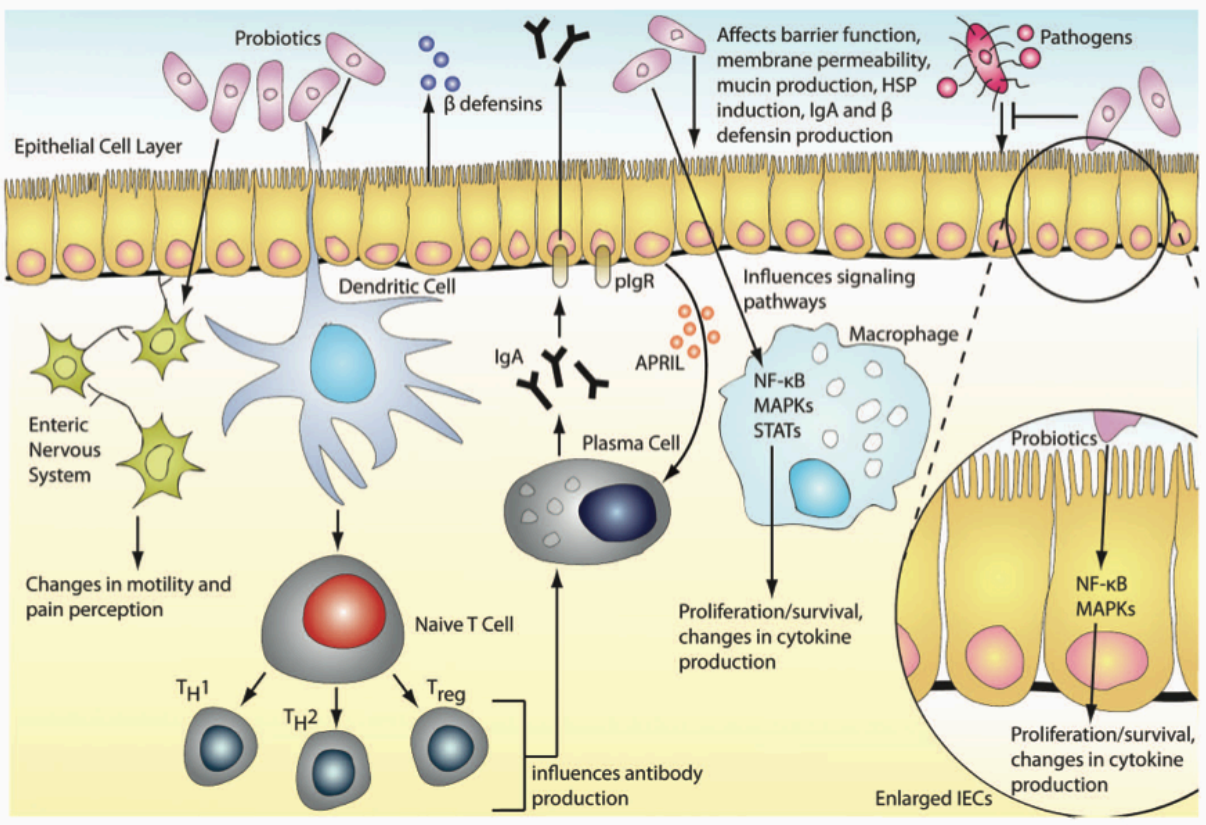


Figure 10. Overall scheme describing the mechanisms of actions of probiotics towards human intestinal cells (from Thomas and Versalovic, 2011).

Even if a wide strain-specific variation in the immune response stimulated by probiotics has been described (Delcenserie et al., 2008), it can be generally regarded that probiotics can interact with the mucosa-associated immune system and bind to epithelial surface receptors, inducing humoral and cellular immune responses. The establishment and maintenance of a well-balanced ratio between pro- and anti-inflammatory cytokines are crucial for the human health. Cytokine secretion by IECs, macrophages and dendritic cells is regulated by probiotics through modulation of key signaling pathways such as NFκB and MAPKs. Changes in these

pathways can also affect proliferation and survival of target cells. Through interactions with dendritic cells, probiotics can additionally influence T cell subpopulations and skew them towards a Th1, Th2 or Treg response (Thomas and Versalovic, 2011).

Several *in vitro* and *in vivo* studies demonstrated two main effects of probiotics on the host immunity: i) strengthening the immunological barrier by stimulating the development and maintaining the state of alert of innate and adaptive immune system; ii) decreasing immune responsiveness to unbalanced inflammatory conditions. Both these health-promoting activities are accomplished through an effective modulation of the balance of pro- and anti-inflammatory cytokines production (Vanderpool et al., 2008). Many probiotic species have been demonstrated to share a relatively common immune pattern, as a reduction in Th2 cytokines (i.e., IL-4, IL-5, IL-6, IL-10, IL-13) or a shift towards Th1-mediated immunity (i.e., IL-2, TNF-α, IFN-γ production).

Prebiotics and human health

At present, prebiotics represent a useful and established dietary approach for influencing the composition of the human gut microbial community. The concept of prebiotics was introduced in 1995 by Gibson and Roberfroid as an alternative way to modulate the gut microbiota. A more recent definition of the term is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health (Gibson et al., 2004).

Generally, prebiotics are oligosaccharides or more complex saccharides that are selectively metabolized by some commensal groups, including species considered to be beneficial for the host. The concept of prebiotics has been recently formalized by the establishment of three scientific criteria that a food ingredient must satisfy to be considered as prebiotic (Gibson et al., 2004): i) resistance to gastric acidity and hydrolysis by mammalian enzymes and

gastrointestinal absorption; ii) substrate of fermentation by intestinal microorganisms belonging to the human microbiota; iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing.

Prebiotics are obtained either by extraction from natural sources, by enzymatic hydrolysis of plant polysaccharides, and by trans-galactosylation reactions catalyzed by bacterial enzymes (Charalampopoulos and Rastall, 2011). Several complex oligosaccharides fulfill the three above mentioned criteria and can be effectively considered as prebiotics. Most promising prebiotics are non-digestible fructo-oligosaccharides (FOS), such as inulin and oligofructose. Inulins are common plant storage carbohydrates which are nutritionally classified as dietary fibers. Inulin-type fructans are present in a range of different plants including wheat, onion, banana, garlic, leek and Agave tequilana (Gomez et al., 2009). Transgalacto-oligosaccharides (GOS) are a mixture of oligosaccharides derived from the enzymatic transglycosylation of lactose, and are among the best characterized prebiotic ingredients (Torres et al., 2010). Besides FOS and GOS, several other potential prebiotic compounds have been identified, such as isomalto-oligosaccharides (IMO), lactosucrose, xylo-oligosaccharides (XOS), soyabean oligosaccharides and gluco-oligosaccharides. However, evidences are still not sufficient for the classification of these oligosaccharides as prebiotics in accordance with the three rules reported above. Recently, the prebiotic potential of oat bran (Kedia et al., 2009) and a new potential IMO-type prebiotic compound, panose (Mäkeläinen et al., 2009), have been also investigated.

Several dietary fibers, including non-starch polysaccharides, whole-grain, cellulose, dextrans, chitins, pectins, β-glucans and waxes have been reported to potentially provide similar beneficial effects as those of inulin-type fructans (Laparra and Sanz, 2010; Costabile et al., 2008; Napolitano et al., 2009).

The biological effect of prebiotics mainly depends on their influence on the gut microbiota composition and metabolism, exerted through a number of different functional properties (Fig. 11), such as increased host absorption of mineral calcium and magnesium, prevention of pathogen adhesion and colonization, modulation of bowel habits, regulation of lipid and glucose metabolism and influence of the intestinal metabolome (Candela et al., 2010; Saulnier et al., 2009; Sherman et al., 2009).

For example, it has been reported that oligofructose supplementation lowers hunger, promotes weight loss and improves glucoregulation in obese and healthy adults (Parnell and Reimer,

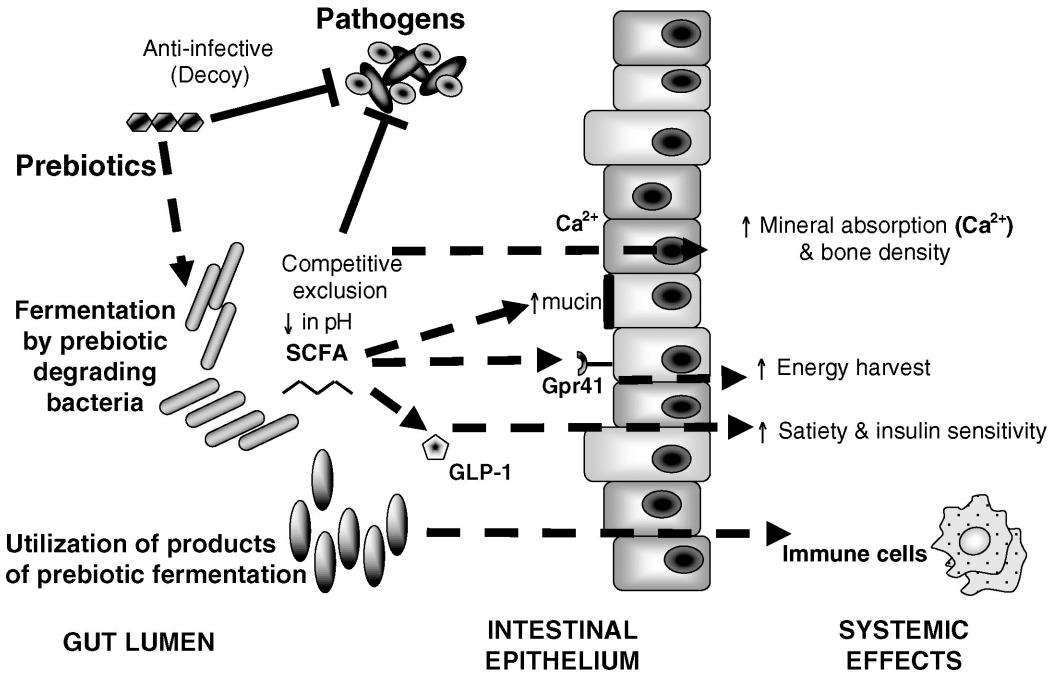


Figure 11. Overall scheme describing the mechanisms of actions of prebiotics towards human intestinal cells (from Saulnier et al., 2009).

2009). The mechanism by which the prebiotics modulation of gut microbiota impacts on the appetite sensation is poorly understood, however it has been suggested that prebiotics could modulate the intestinal microbiota, lowering the high-fat diet-induced LPS endotoxaemia and systemic and liver inflammation (Cani et al., 2009).

Very recently, it has been further demonstrated that prebiotic-enriched pasta could be a useful dietary tool to manipulate gut microbiome-mediated well-being endpoint (Russo et al., 2010; 2011).

The influence of prebiotics on host immunity, defense and inflammatory processes represents another interesting biological aspect of these food ingredients. Indeed, prebiotics effects may influence the immune system both directly or indirectly, through different proposed mechanisms, which have been extensively reviewed by Roberfroid et al. (2010): i) immune modulation of prebiotics can result from the compositional modification of the intestinal microbiota upon their ingestion and fermentation. This can lead to increased or depleted concentrations of peculiar microbial genus or species, which may change the overall immuno-interactive profile of the microbiota; ii) microbial products, mainly SCFAs, may interact with immune cells and enterocytes and modify their activity. In fact, that SCFAs modulate cytokine expression in intestinal epithelial cells is a matter of fact, since it has been demonstrated that they are able to differentially affect pro-inflammatory IL-2 and IFN-γ and immunoregulatory IL-10 production. Furthermore, it has been recently proposed the importance of SCFAs ligation to GPR43 to maintain intestinal homeostasis; iii) the potential direct ligation of pattern recognition receptors on immune cells by prebiotic carbohydrate structures may result in immunomodulation.

PROJECT OUTLINE

CHAPTER 2 - PROJECT OUTLINE

The role of the human gut microbiota in impacting host's health has been widely studied in the last decade. In particular, the development of "omics" techniques allowed researchers to integrate in a holistic view the complementary knowledge deriving from studies on the composition, functional activity and metabolism of the billions of microbial cells which are thriving in our intestine (Maccaferri et al., 2011).

Notably, it has been recently shown that diet and nutritional status are among the most important modifiable determinants of human health. Through the plethora of presumptive mechanisms, microbiota-mediated processes are thought to have a relevant role (Kau et al., 2011).

At present, probiotics and prebiotics represent a useful and established dietary approach for influencing the composition and activity of the human gut microbial community. Notably, the consumer market for probiotics, prebiotics and functional foods is > 1.4 billion euros, with an estimated annual growth of about 7-8% in the 2008-2013 period. (Saxelin, 2008).

The present study is composed of two main sections aimed at elucidating the probiotic potential of the yeast strain *K. marxianus* B0399, as well as the putative prebiotic activity ascribable to four different flours, naturally enriched in dietary fibres content.

Firstly, several potential probiotic traits of *K. marxianus* B0399 were investigated by using *in vitro* assays, including adhesion and immune modulation. The effect of the administration of 10^7 CFU/day of *K. marxianus* B0399 on the composition and metabolic activity of the human intestinal microbiota was investigated in a 3-stage continuous-culture system simulating the human colon. The promising results demonstrating that this lactic yeast strain possesses a

number of beneficial and strain-specific probiotic properties (Maccaferri et al., 2012), substantiated further *in vivo* studies. In particular, we investigated if the daily administration of a novel probiotic yoghurt containing *B. animalis* subsp. *lactis* Bb12 and *K. marxianus* B0399 could impact on the biostructure of IBS microbiota, modulating its composition towards a healthy-like pattern. Indeed, this yoghurt was demonstrated to have beneficial effects in the management of IBS symptoms (Lisotti et al., 2011).

Additionally, we investigated the impact of four flours (Wholegrain rye, wholegrain wheat, chickpeas and lentils 50:50, and barley milled grains), characterised by a naturally high content in dietary fibres, on the intestinal microbiota composition and metabolomic profiles. These studies combined molecular and cellular analysis, performed in order to evaluate composition the activity of the human colonic microbiota, with a NMR metabolomics approach for determining the impact of potential prebiotic fibres on the intestinal metabolome.

KLUYVEROMYCES MARXIANUS B0399:
IN VITRO AND *IN VIVO* CHARACTERISATION OF A NOVEL PROBIOTIC YEAST ON THE HUMAN GUT ECOSYSTEM

CHAPTER 3 - *IN VITRO* EVALUATION OF THE PROBIOTIC ACTIVITY OF *K. MARXIANUS* B0399

Increasing evidence is substantiating the utilisation of beneficial microbes in functional foods, dairy products or other dietary supplements aimed at maintaining and promoting human health (Jankovic et al., 2010). Probiotics have been demonstrated to exert health-promoting effects through several proposed mechanisms, including SCFAs production and enhancement of the barrier function of the intestinal epithelium, suppression of growth and binding of pathogenic bacteria, alteration of the immune activity of the host (Aragon et al., 2010; Thomas and Versalovic, 2011; Ventura et al., 2009). Adhesion to the intestinal epithelium is an important requisite for allowing probiotics to modulate the immune system. Since adhesion ability is strongly strain-dependent, an evaluation of this characteristic is required as a selection criterium for novel probiotics (Collado et al., 2009). Probiotics can interact with the mucosa-associated lymphoid tissues and bind to epithelial surface receptors, inducing humoral and cellular immune responses. The establishment and maintenance of a well-balanced ratio between pro- and anti-inflammatory cytokines are crucial for the human health. Therefore, studying the dynamic cytokines modulation (Tab. 1) elicited by a microorganism represents a hot topic in the selection of novel probiotic strains. A wide strain-specific variation in the immune response stimulated by probiotics has been described, and several *in vitro* cell models have been developed to evaluate their immunomodulatory effects (Delcenserie et al., 2008).

Immune mediators		Major functions
Tumour necrosis factors	TNF-α, TNF-β	Activation of immune cells, induction of apoptosis, pro-inflammatory functions
Interleukins	IL-1 α/β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17.	Regulation of immune cell function and activation
Transforming growth factors	TGF-β	Inhibition of cell growth, anti-inflammatory, induction of IgA secretion
Interferons	IFN-α/β/γ	Antiviral activity, inhibition of tumour cell growth, activation of immune cells
Chemokines	CCL2, -3, -4, -5, CXCL8, -10	Chemotaxis and activation of immune cells
Colony-stimulating factors	GM-CSF, G-CSF, IL-3	Growth and differentiation of hematopoietic cells
Growth factors	EGF, FGF, PDGF	Growth and differentiation of cells

Table 1. Summary of the main immune mediators

Even if these cellular models lack the immune system complexity, they allow to elucidate mechanisms involved in bacterial sensing by human colonocytes and immune competent cells (Boivirant et al., 2007). Functional foods commonly contain specific lactic acid bacteria strains, mainly belonging to *Bifidobacterium* or *Lactobacillus* genera. Less frequently used are strains of *Propionibacterium freudenreichii*, bacilli or yeasts (Wassenaar and Klein, 2008). *Kluyveromyces marxianus* is a lactic yeast isolated from different dairy products, mainly kefir (Bolla et al., 2011; Farnworth, 2005; Jianzhong et al., 2009). While the importance of this species in food development and fermentation is well documented, characterisation of its putative probiotic activities is very limited (Kumura et al., 2004; Romanin et al., 2011).

Kumura et al. (2004) evaluated a limited number of probiotic activities (adhesion to human enterocyte-like Caco-2 cells, resistance in acidic conditions and stimulation of IL-8 synthesis) in yeasts of dairy origin, testing 8 species belonging to *Candida*, *Debaryomyces*, *Kluyveromyces*, *Yarrowia* and Saccharomyces genera, isolated from commercial blue cheese and kefir. Romanin et al. (2010) attempted to to select lactic yeast from kefir able to down-regulate intestinal epithelial innate response.

Since the interest of the food industry in the selection of novel candidate probiotic strains, we evaluated for the first time the probiotic potential of *K. marxianus* B0399, a strain isolated from whey and curds of cow's milk and deposited (deposit number MUCL 41579) at Belgian Coordinated Collection of Microrganism (BCCM). This strain is of particular interest for several reasons: (i) it is included in the EFSA list of Qualified Presumption of Safety (QPS) biological agents added to food and feed (EFSA Panel on Biological Hazards, 2010); (ii) it is included in different functional foods, currently marketed in several countries; (iii) it is capable of survival during gastric transit, maintaining its vitality and fermentation capacity (Mustacchi et al., 2010).

Here, *K. marxianus* B0399 was assessed for its ability to adhere to the human enterocyte-like Caco-2 cells. Furthermore, the capacity to modulate the production of 27 immune-mediators (cytokines, chemokines and growth factors) in Caco-2 cells and peripheral blood mononuclear cells (PBMCs) was evaluated. Finally, the effect of the daily administration of 10^7 CFU of *K. marxianus* B0399 on the faecal microbiota of 2 individuals affected by constipation-Irritable Bowel Syndrome (IBS) was investigated using a continuous culture system simulating the human colon (Box n. 4). Indeed, IBS patients are generally considered an appropriate study group to support claims on gastrointestinal discomfort intended for general population (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2011). The 3-stage continuous culture colonic model system used in this study (Fig. 12) provides a controlled environment that can

be maintained in a steady state and that simulates the complexity and diversity of the microbiota. Therefore, it represents a useful tool for monitoring the ecology and the metabolic activities of colonic microbiota in relation to different external perturbations (Bahrami et al., 2011; Maccaferri et al., 2010; Macfarlane et al., 1998).

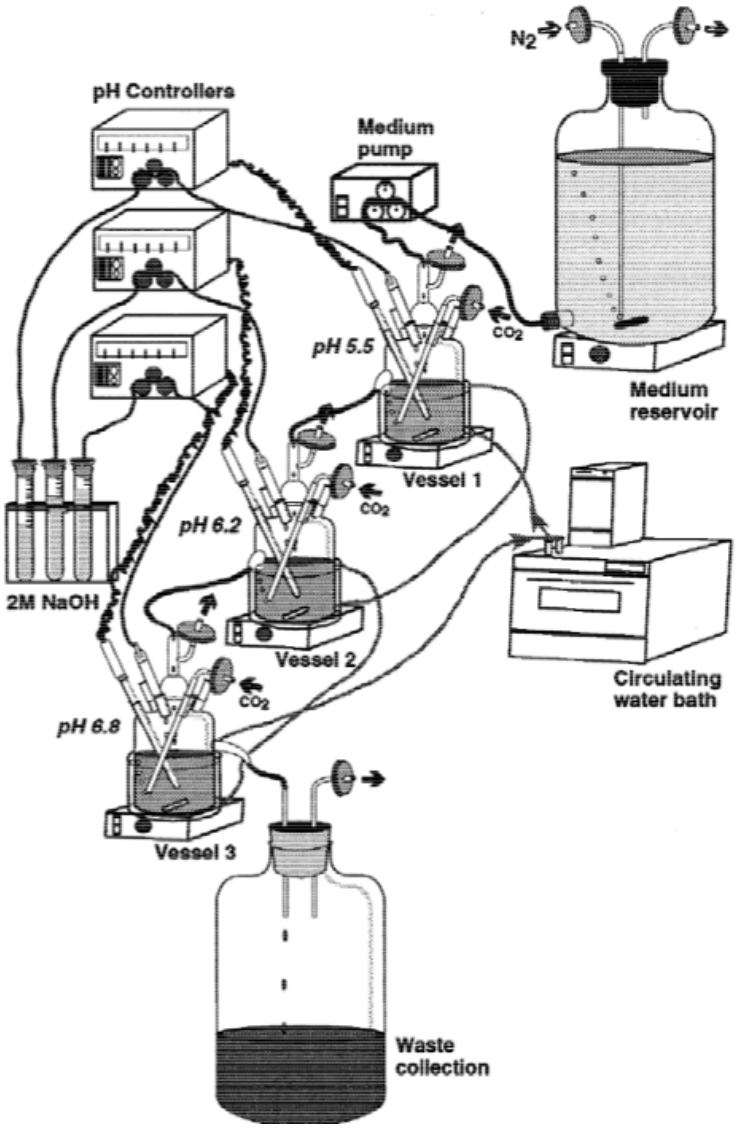


Figure 12. Scheme of the three-stage continuous culture system simulating the human colon, used in this study.

BOX N. 4 - *IN VITRO* MODEL TO STUDY THE GUT MICROBIOTA

Access to human materials other than faeces, such as gut content and tissues, is often restricted for practical and ethical reasons. At the same time, animal digestive physiologies differ from humans and only very recently humanised mice, harbouring a human-like microbiota have been introduced. Therefore, the insurmountable problem of inaccessibility of the digestive tract in healthy people stimulated scientists to use gut peculiar characteristics to develop and improve several *in vitro* models, involving the use of pure cultures, defined mixed cultures and faecal material. These models can range from simple batch fermentation system, to sophisticated pH controlled multistage continuous culture systems. *In vitro* models are tools of striking interest for the investigation of microbe-mediated processes that occur in the colonic lumen. Especially, multistage continuous cultures facilitate long-term studies and allow perturbations to the microbiota to be investigated under steady-state conditions. According to Longland (1991), an adequate gastrointestinal *in vitro* model should have the following characteristics: i) physiological quantities of enzymes should be used in sequence; ii) the pH should allow the activation of enzymes and other cofactors (i.e., bile salts and coenzymes); iii) digestive end products should be removed from the system; iv) at each stage, digesta should be adequately mixed; v) the transit rate should simulate the representative host species.

The simplest *in vitro* system to evaluate response of the gut microbiota to exogenous factors is the **batch system**. A batch system consists of a single, self-contained vessel. This method is rapid, inexpensive but does not allow addition or removal of nutrients from the vessel.

Gibson and colleagues (1988) developed a **three-stage continuous culture system**, designed to reproduce spatial, temporal, nutritional, and physicochemical characteristics of the microbiota in the proximal and distal colons. This *in vitro* colonic model was validated on the basis of chemical and microbiological measurements on intestinal contents obtained from human sudden death victims (MacFarlane et al., 1998). This model does not take into account host factors such as intestinal secretions, gut immunological events, or the absorption of bacterial metabolites, however it provides a useful and relatively inexpensive tool for modelling the ecology and metabolic activities of large intestine bacteria, under different environmental conditions.

Frequent sampling at various locations of the model and the possibility to vary several parameters that influence the fermentative processes in the colon (i.e. pH and food transit time), enable detailed mechanistic insight into, such as the fermentation of non-digestible carbohydrates or administration of probiotics, prebiotics and other xenobiotics. This colon model consists of three vessels arranged in series, with respective operating volumes of 280, 300 and 320 mL. Vessels feed into each other sequentially, thus representing the proximal, transverse and distal regions of the human colon in terms of pH and nutrient availability. Furthermore, to reproduce the pH characteristics of the large gut, each vessel has a different pH, respectively of 5.5, 6.2, and 6.8. A mixture of polysaccharides and proteins is used as carbon and nitrogen sources, and each fermentor is magnetically stirred and maintained at a temperature of 37°C and under anaerobic conditions, in order to be as much as possible near to physiological conditions.

Culture conditions of *K. marxianus* B0399

K. marxianus B0399 was routinely grown aerobically at 37°C in MV2 broth (lactose, 40 g/L; casein, 20 g/L; peptone, 7.5 g/L; yeast extract, 1.5 g/L). The ability of *K. marxianus* B0399 to survive in the colonic model conditions was assessed incubating 7.0 log CFU/mL of actively growing culture in complex Colonic Model Growth Medium (CMGM) (Macfarlane et al., 1998), at 37 °C in anaerobic conditions for 24 h.

The resistance of the yeast strain in an environment simulating the upper gastrointestinal tract was further *in vitro* evaluated, as previously described by Maragkoudakis et al. (2006). Briefly, actively growing culture was harvested (10,000 x *g* for 5 min, at 4 °C) and washed twice in PBS. Resistance to the environmental condition of the stomach was assessed resuspending the cells pellet (final concentration 6.0 – 7.0 log CFU/mL) in 0.1 mol/L PBS adjusted by HCl to pH 2, containing pepsin (3 mg/mL, Sigma-Aldrich), and evaluating the viable colony counts after 3 h of incubation at 37 °C. Bile salts tolerance was tested assessing colony viability after 3 h incubation in MV2 broth, supplemented by 0.3% (w/v) Oxgall (Sigma).

Eukaryotic cells culture conditions

The human enterocyte-like Caco-2 cells were grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with 1.5 g/L glucose, 10% heat-inactivated fetal calf serum (Cambrex), 1% non-essential amino acids (Sigma), penicillin (50 IU/mL) and streptomycin (50 µg/mL), at 37 °C in an atmosphere of 5% CO_2. The growth medium was changed to fresh medium without the addition of antibiotics for the last 24 h incubation prior to performing the immunoassay and the adhesion assays.

The human colon adenocarcinoma HT29 cells were grown in DMEM supplemented with 10% (w/v) fetal bovine serum, penicillin (50 IU/mL) and streptomycin (50 µg/mL), at 37 °C in an atmosphere of 5% CO_2.

PBMCs were isolated from healthy volunteers by density gradient centrifugation (Lymphoprep, Nycomed Pharma). Cells were re-suspended in Roswell Park Memorial Institute-1640 culture medium [RPMI-1640 (Life Technologies)] supplemented with 10% (w/v) foetal bovine serum (Thermo Fisher Scientific Inc.) and 0.23 mM sodium pyruvate solution (Sigma). PBMCs (10^6 cells/mL) were cultured at 37 °C in a humidified atmosphere, containing 5% CO_2.

Adhesion of *K. marxianus* B0399 to Caco-2 cells detected by qPCR

Adhesion to Caco-2 cells of *K. marxianus* B0399 was evaluated by quantification of Caco-2-bound microorganisms via qPCR, as reported by Candela and colleagues (2008). Stationary-phase-grown cells of the yeast and bacteria strains were washed and re-suspended at the cell density of approximately 8 Log CFU/ml in DMEM. Caco-2 cells were washed with DMEM and 1 mL of DMEM, containing the yeast/bacterial suspension, was added. After incubation for 1.5 h at 37 °C under humidified atmosphere, unattached yeast or bacteria were removed by washing the monolayers four times with sterile PBS. After detachment of Caco-2 cells

from the plastic surface by treatment (15 min at 37 °C) with 200 µL trypsine/EDTA (Cambrex Bio Science) per well, Caco-2 cells and adhesive yeast or bacteria were transferred into a 1.5 mL-reaction tube. To quantify microbial cells by qPCR, cell suspensions were boiled for 5 min and, after mixing, an aliquot of 20 µL was transferred into an 0.2 mL-reaction tube and incubated for 10 min at room temperature with 3.8 µL of Trypsin Inhibitor solution (Type I-S from Soybean, 1 mg/mL in H_iO). Strongly-adhesive enterotoxigenic *Escherichia coli* H10407 and mildly-adhesive *Leuconostoc mesenteroides* C5 were used as reference bacterial strains. Quantification of the reference bacterial strains was performed with *E. coli* species-specific primer set ECO-1/ECO-2 (Wang et al., 1996) and LAB-specific PCR primer set Bact-0011f/ Lab-0677r (Heilig et al., 2002), whereas yeast-specific primer set NL1/LS2 (Cocolin et al., 2000) was used to quantify *K. marxianus* B0399. qPCR was performed in a LightCycler instrument (Roche) and SYBR Green I fluorophore was used to correlate the amount of PCR product with the fluorescence signal. Quantification of bacterial and yeast DNA was carried out using standard curves made from known concentrations of genomic DNA from the reference bacterial strains and *K. marxianus* B0399. The experimental protocol consisted of the following programs: (i) starting pre-incubation at 95 °C for 10 min; (ii) amplification including 30 cycles of 4 steps each at the temperature transition time of 20 °C/s: denaturation at 95 °C for 15 s, annealing at 63 °C (Bact-0011f/Lab-0677r) or 60 °C (ECO-1/ECO-2 and NL1/LS2) for 25 s, extension at 72 °C for 30 s, and fluorescence acquisition at 85 °C (Bact-0011f/Lab-0677r and NL1/LS2) or 88 °C (ECO-1/ECO-2) for 5 s; (iii) melting curve analysis: heating at 20 °C/s to 95°C; cooling at 20 °C/s to 60 °C with 15 s hold, and then heating 0.2 °C/s until 99 °C. Chromosomal DNA of the strains used as standards was extracted by using DNeasy Tissue Kit (Qiagen) and serially diluted from 10^6 to 10^3 CFU/µL. The data reported represent mean values obtained in 3–5 independent experiments. Each experiment was performed in duplicate.

Immunoassay

K. marxianus B0399 cells, corresponding to a concentration of 1 x 10^6 CFU/mL, were applied to confluent Caco-2 cells or PBMCs and incubated at 37 °C for 24 h. Unstimulated cells were used as a negative control, whereas LPS (1 μg/mL, Sigma) was used to stimulate eukaryotic cells. After incubation, supernatants from Caco-2 and PBMCs cultures were collected, centrifuged at 400 x *g* for 15 min at 4 °C and used to determine levels of several immune-mediators by using a multiplexed bead immunoassay. Caco-2 and PBMCs cells were checked for viability by Trypan blue exclusion.

The concentration of 27 immune-mediators (Table 2) was measured using the human ultrasensitive cytokine 27-plex antibody bead kit (Bio-Rad). The assays were performed in 96-well filter plates, as previously described (Vignali, 2000). The concentration of the samples was estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/mL after adjusting for the dilution factor (Bio-Plex Manager software version 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve. The intra-assay CV averaged 17%. Experiments were performed in triplicate. For each single determination, 50 beads were read and standard deviation was calculated.

Immune mediators	Chemical class
Interleukin-(IL): IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17. Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNFα)	***Cytokines***
Monocyte Chemotactic Protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α), Macrophage Inflammatory Protein-1β (MIP-1β), Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), Eotaxin, IL-8, Interferon-γ-induced Protein-10 (IP-10)	***Chemokines***
Platelet-derived Growth Factor-BB (PDGF-BB), Fibroblast Growth Factor (FGF basic), granulocyte colony-stimulating factor (G-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Vascular Endothelial Growth Factor (VEGF)	***Growth factors***

Table 2. List of the immune-mediators evaluated in the present study

Three-stage continuous culture colonic model system

The three-stage continuous culture model of the human colon comprised of 3 glass fermenters of increasing working volume, simulating the proximal (V1, 280 mL), transverse (V2, 300 mL) and distal colon (V3, 320 mL). The 3 fermenters connected in series were kept at 37°C, pH was stably maintained at 5.5 (V1), 6.2 (V2) and 6.8 (V3) and anaerobic conditions were ensured by continuously sparging with O_2-free N_2.V1 was fed by means of a peristaltic pump with CMGM (Macfarlane et al., 1998).

Human faecal samples were collected on site, kept in an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2) and used within a maximum of 15 min after collection. This experiment was carried out in duplicate using faecal samples from two different volunteers suffering from constipation IBS. None of the volunteers had received antibiotics or probiotics for at least 3 months before sampling. A 1:5 (w/w) faecal dilution in anaerobic PBS [0.1 M PBS (pH 7.4)] was prepared and the samples were homogenised in a stomacher (Seward Ltd.) for 2 min. Each stage of the colonic model was inoculated with 100 mL faecal slurry. Total system transit time was set at 72 h, according to mean retention time of adults suffering from constipation IBS. Following inoculation, the colonic model was run as a batch culture for a 24 h period in order to stabilise bacterial populations prior to the initiation of medium flow. After 24 h (T0) the medium flow was initiated and the system was run for 8 full volume turnovers to allow for steady state to be achieved (SS1). Taking into account the operating volume (900 mL) and the retention time (72 h) of the colonic model system, 3-5 x 10^7 CFU of actively growing *K. marxianus* B0399 were added daily to V1. The yeast strain was added to the system as described for a further 8 volume turnovers upon which steady state 2 (SS2) was achieved. Each steady state was confirmed through sampling on three consecutive days for SCFAs and fluorescence in situ hybridization (FISH) analyses. Samples for FISH were immediately fixed in 4% paraformaldehyde as previously described (Martín-Peláez et al.,

2008). Samples for HPLC and cytotoxicity analysis were centrifuged and supernatants were frozen immediately, whereas cell pellets were resuspend in PBS/glycerol (1:1) and stored at -20 °C prior to proceed with DNA extraction.

Evaluation of the colonic microbiota composition by FISH, PCR-DGGE and qPCR

Concentration of the main intestinal bacterial groups in samples from the colonic model system was evaluated by FISH, as previously described by Martín-Peláez et al. (2008). The probes used are reported in Annex 1 and were commercially synthesised and 5'-labelled with the fluorescent Cy3 dye (Sigma).

Dynamics of yeast population during the study was assessed by PCR-DGGE and qPCR. Frozen samples recovered from the colonic model system were thawed and aliquots (250 μL) were processed for DNA extraction as previously described by Maccaferri et al. (2010). Approximately 250 nucleotides of the 5'-end region of the 26S rRNA gene was amplified by PCR using the yeast-universal primer set NL1 (or GC-clamped NL1 for PCR-DGGE) and LS2, according to Cocolin et al. (2000). The PCR-DGGE experimental protocol was slightly modified by performing annealing at 56 °C for 25 s and extension at 72 °C for 30 s, in order to prevent cross-amplification of bacterial DNA. Band identity was confirmed by comparison of the position in the gel length with those of reference yeast DNA, as well as by band excision, re-amplification and sequencing. qPCR was performed as previously described, using standard curves made from known concentrations of the genomic DNA of *K. marxianus* B0399, in order to quantify modifications in concentration of yeasts along the experiment.

Determination of short-chain fatty acids concentration by HPLC

Samples from each vessel of the colonic model system were centrifuged at 13,000 x *g* for 10 min to remove bacterial cells and any particulate material. SCFAs (acetate, propionate, butyrate) and lactic acid concentrations were determined by HPLC on an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad). Degassed 5 mM H_2SO_4 was used as eluent at a flow rate of 0.6 mL/min and an operating temperature of 50 °C. Organic acids were detected by UV at a wavelength of 220 nm. Sample quantification was carried out using calibration curves standard for lactate, acetate, propionate and butyrate at concentrations of 12.5, 25, 50, 75 and 150 mM. Internal standard of 20 mM 2-ethylbutyric acid was included in the samples and external standards.

Modulation of HT29 cells growth by *K. marxianus* B0399

The influence of colonic model supernatants, recovered before and after administration of *K. marxianus* B0399, on the growth and survival of the human colon carcinoma cell line HT29 was determined using the growth curve assay, as previously described by Maccaferri et al. (2010). Results are expressed as EC_{50}, which represents the effective concentration of colonic model supernatants resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions compared to the growth of untreated cells.

Statistical analysis

All data were analysed by one-way ANOVA, using Tukey's post-test analysis when the overall P value of the experiment was below the value of significance ($P < 0.05$). An additional paired t-test was applied in order to assess the significance of results of single pairs of data. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software).

Adhesion of *K. marxianus B0399* to Caco-2 cells

Adhesion ability of *K. marxianus* B0399 to Caco-2 cells was evaluated by qPCR (Fig. 13). Notably, *K. marxianus* B0399 showed adhesion value of 4.13 x 10^3 cells/100 Caco-2 cells, whereas reference bacterial strains *L. mesenteroides* C5 (mildly-adhesive bacterial strain) and *E. coli* H10407 (strongly-adhesive bacterial strain) showed adhesion values of 7.61 x 10^2 and 10.56 x 10^4 cells/100 Caco-2 cells, respectively. According to Candela et al. (2008), who defined strongly adhesive strains as those with more than 40 cells adhered to one Caco-2 cell, *K. marxianus* B0399 can be classified as a strongly adhesive strain.

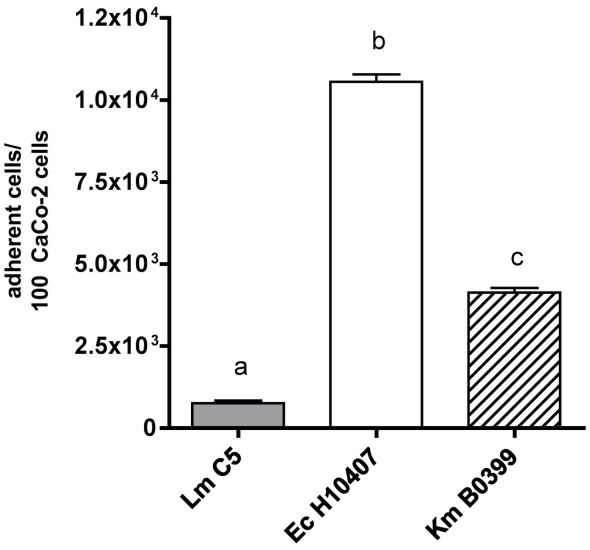


Figure 13. Adhesion of *K. marxianus* B0399 (Km B0399) to Caco-2 cells, as evaluated by qPCR. The strongly adhesive enterotoxigenic *E. coli* H10407 (EcH10407) and mildly adhesive *L. mesenteroides* C5 (Lm C5) were used as control. Measurements were performed in triplicate. Results are means (number of cells of the target strain bound to 100 Caco-2 cells) ± SEM.

Immunomodulatory activity of *K. marxianus* B0399 towards PBMCs and Caco-2 cells

The ability of *K. marxianus* B0399 to modulate the secretion of 27 immune-mediators in PBMCs and Caco-2 cells was tested. Unstimulated cells were used as negative control, whilst LPS-stimulated cells were used as positive control.

The incubation of PBMCs with *K. marxianus* B0399 provoked a marked increase in concentration of the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, MIP-1α and TNF-α and a moderate yet significant increase in concentration of anti-inflammatory cytokine IL-1Ra. Conversely, when LPS was used to trigger an inflammatory response, co-incubation with *K. marxianus* B0399 elicited a significant decrease in the concentration of pro-inflammatory TNF-α, IL-6, and MIP-1α, whereas IL-1β was significantly increased in concentration. No significant variations were detected for IFN-γ and IL-1Ra after the co-incubation (Fig. 14).

The incubation of Caco-2 cells with *K. marxianus* B0399 provoked a significant decrease of secretion of the pro-inflammatory chemokine IP-10. When *K. marxianus* B0399 was co-incubated with LPS, it induced a significant decrease of secretion of the pro-inflammatory cytokines IP-10, IL-8, IL-12, and IFN-γ (Fig. 15).

The production of the other immune modulators by PBMCs and Caco-2 cells was not significantly modulated by *K. marxianus* B0399 in all the tested conditions.

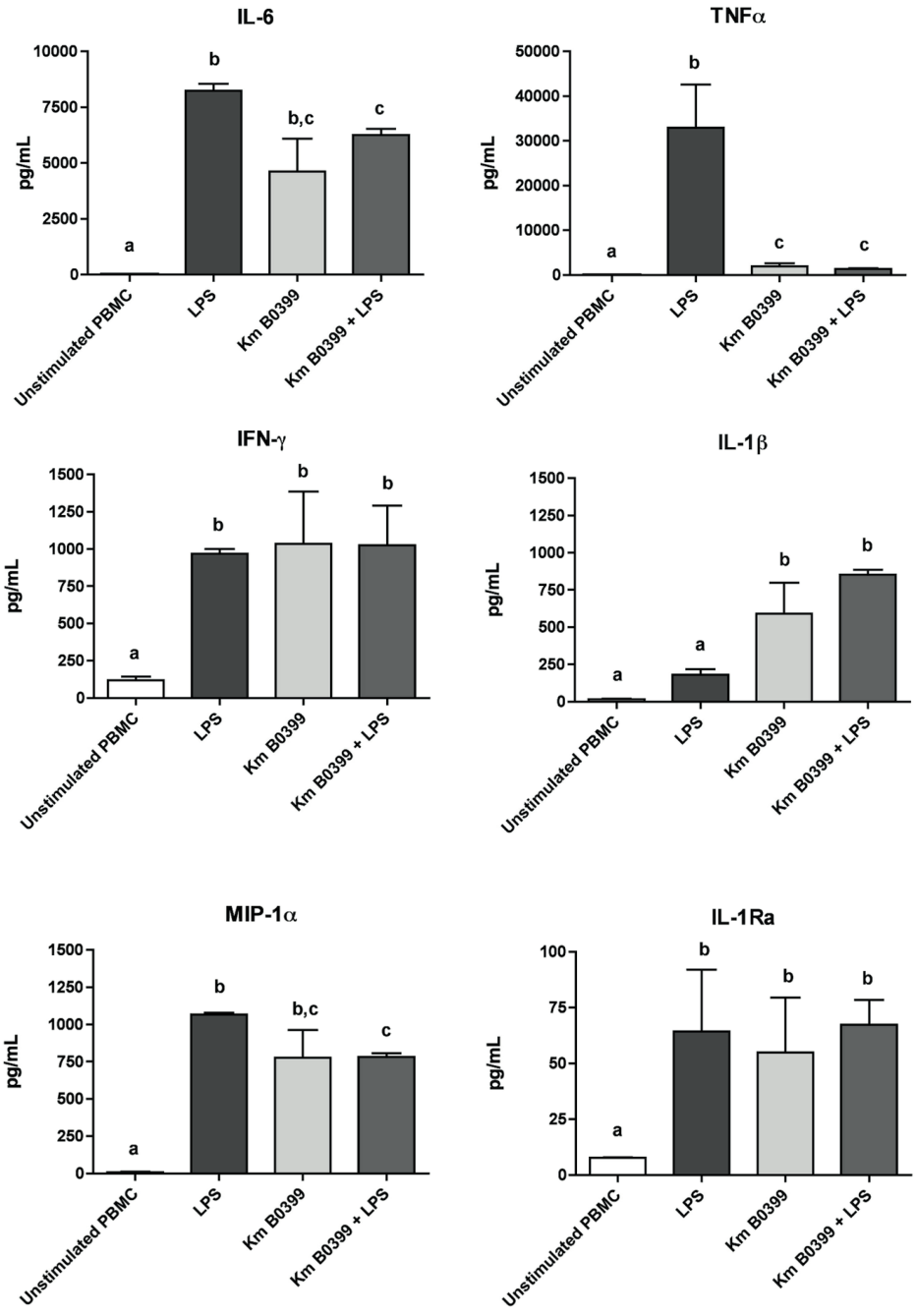


Figure 14. Levels of immune-mediators secreted by PBMCs, after stimulation with LPS, *K. marxianus* B0399 (Km B0399) and co-stimulation with LPS and yeast strain (Km B0399 + LPS). Measurements were performed in triplicate. Results are means (pg of immune-mediators per ml of cultural supernatant) ± SEM. Bars not sharing a common letter are significantly different at a confidence level of $P < 0.05$.

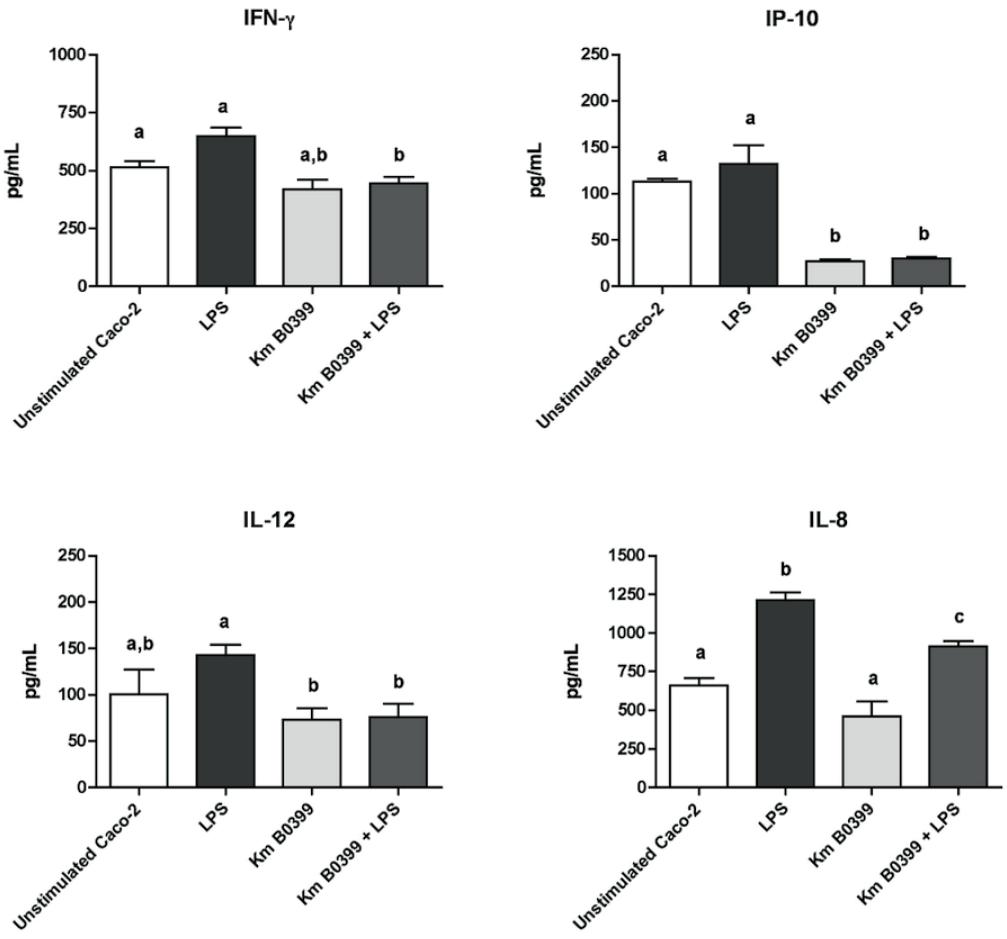


Figure 15. Levels of immune-mediators secreted by Caco-2 cells, after stimulation with LPS, *K. marxianus* B0399 (Km B0399) and co-stimulation with LPS and yeast strain (Km B0399 + LPS). Measurements were performed in triplicate. Results are means (pg of immune-mediators per ml of cultural supernatant) ± SEM. Bars not sharing a common letter are significantly different at a confidence level of $P < 0.05$.

Survival of *K. marxianus* B0399 in simulated gastrointestinal conditions

K. marxianus B0399 was confirmed to survive in gastric simulated conditions, since incubation for 3 h at pH 2 in presence of pepsin provoked a moderate decrease of yeast viability, from an initial concentration of 6.90 log CFU/mL to a final value of 4.97 log CFU/mL. Similarly, survival was maintained when the strain was incubated for 3 h with physiological concentration of bile salts, with a slight decrease from 6.96 log CFU/mL to 6.63 log CFU/mL. *K. marxianus* B0399 was further able to grow anaerobically in the colonic model system medium CMGM, reaching a final concentration of 8.38 log CFU/mL after 24 h (data not shown).

Impact of *K. marxianus* B0399 on the colonic microbiota composition

Total yeasts population was evaluated in each vessel of the colonic model system before (SS1) and after (SS2) the daily administration of *K. marxianus* B0399 by PCR-DGGE. PCR-DGGE, whose sensitivity (~10^5 yeast cells/mL) was not sufficient to detect yeasts in the fermentation system before the intervention, confirmed the presence of a clear band corresponding to *K. marxianus* in V1 and V2 at SS2 (99% identity with *K. marxianus* 13MCHS 26S ribosomal RNA gene, Annex 2). Furthermore, qPCR analysis confirmed that the total yeast population in V1, V2 and V3 at SS1 was below the detection limit of the method (2.5 x 10^2 CFU/mL), whilst at SS2 yeasts reached the following concentrations: V1, 3.7 ± 0.9 x 10^7 CFU/mL; V2, 6.1 ± 0.6 x 10^4 CFU/mL; V3, < 2.5 x 10^2 CFU/mL.

The concentration of the main bacterial groups constituting the core of the human intestinal microbiota was assessed before and after supplementation of *K. marxianus* B0399 by FISH (Fig. 16).

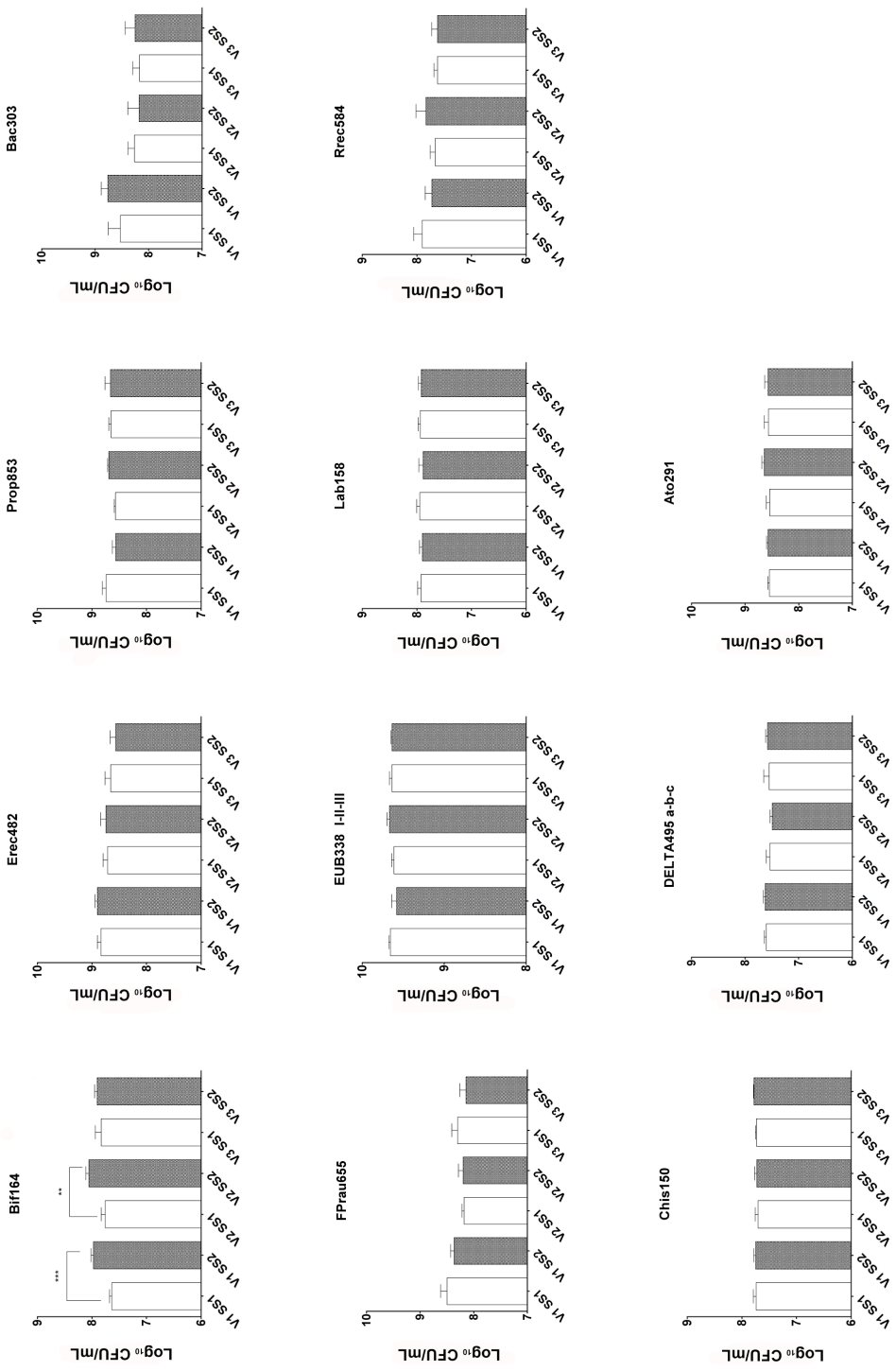


Figure 16. Bacterial groups detected by FISH in the culture broth recovered from each vessels (V1, V2 and V3) of the colonic model system before (SS1) and after (SS2) the daily administration of *K. marxianus* B0399. Results are reported as mean of the data of two colonic models (Log10 cells/mL) ± SEM. For each colonic model, measurements were performed in triplicate at SS1 and SS2. $^{**}P < 0.01$; $^{***}P < 0.001$.

Yeast administration did not mediate any significant modification in the total bacteria counts (EUB338 I-II-III) during the intervention. FISH analysis showed that *Clostridium* cluster XIVa (Erec482) and cluster IX (Prop853) were the predominant bacterial groups in the colonic microbiota, and that *K. marxianus* B0399 addition did not significantly influence ($P > 0.05$) their concentration. A similar behaviour was demonstrated for *Bacteroides* (Bac303), *Faecalibacterium prausnitzii* (Fprau655), the subdominant lactic acid bacteria (Lab158), *Roseburia intestinalis-Eubacterium rectale* group (Rrec584), *Clostridium* cluster I and II (Chis150), *Atopobium* cluster (Ato291) and δ-*Proteobacteria* (DELTA495 a-b-c), whose concentrations were stably maintained during the study.

Notably, administration of *K. marxianus* B0399 provoked a significant increase in bacteria belonging to the health-promoting genus *Bifidobacterium* (Bif164) in the first and second stages of the colonic model system (7.57 to 7.96 log CFU/mL in V1, $P = 0.0004$; 7.78 to 8.12 log CFU/mL in V2, $P = 0.009$).

Impact on SCFAs production by *K. marxianus* B0399

SCFAs (acetate, propionate and butyrate) and lactic acid were detected and quantified by HPLC in the three different stages of the colonic model systems, at SS1 and SS2.

The administration of *K. marxianus* B0399 induced a significant increase of acetate (64.58 to 76.02 mM, $P = 0.02$) and propionate (57.42 to 70.16 mM, $P = 0.0005$) over the course of the experiment. In particular, acetate increased significantly in the first stage of the colonic model system, whereas propionate increased significantly in the first and second stages of the colonic model system (Fig. 17). Conversely, no significant modification in lactate and butyrate concentrations occurred.

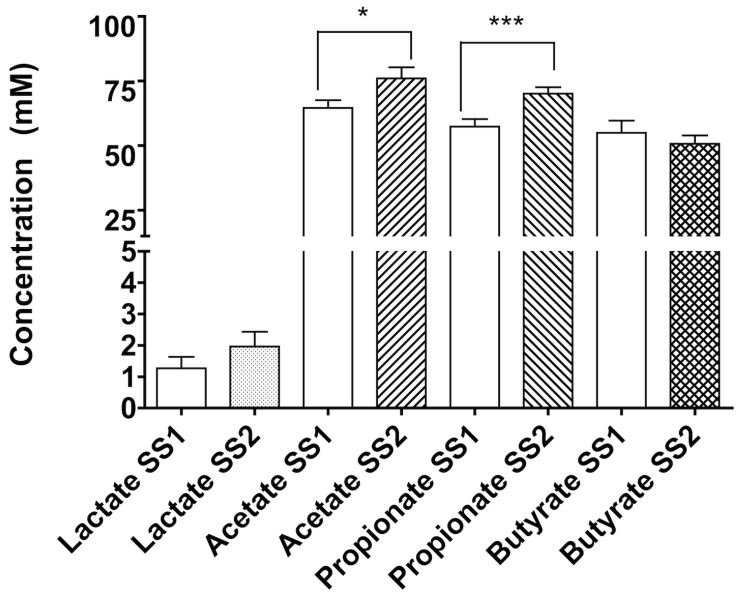


Figure 17. Short-chain fatty acids concentrations in the culture broths recovered from each vessels (V1, V2 and V3) of the colonic model before (SS1) and after (SS2) the daily administration of *K. marxianus* B0399, as assessed by HPLC. Results are reported as mean of the data of two colonic models (mM) ± SEM. For each colonic model, measurements were performed in triplicate at SS1 and SS2. *$P < 0.05$; ***$P < 0.001$.

Cytotoxic effects of colonic model supernatants

EC_{50} was used to compare the effect of colonic model supernatants, before and after the administration of *K. marxianus* B0399, on HT29 cell growth (Fig. 18). No significant changes between EC_{50}(SS1) and EC_{50}(SS2) were found in the second and third stages of the colonic model system. Conversely, V1 colonic model supernatants after the administration of the yeast strain were significantly less cytotoxic than at SS1 (EC_{50} SS1: 3.35 vs. SS2: 4.23, $P < 0.05$).

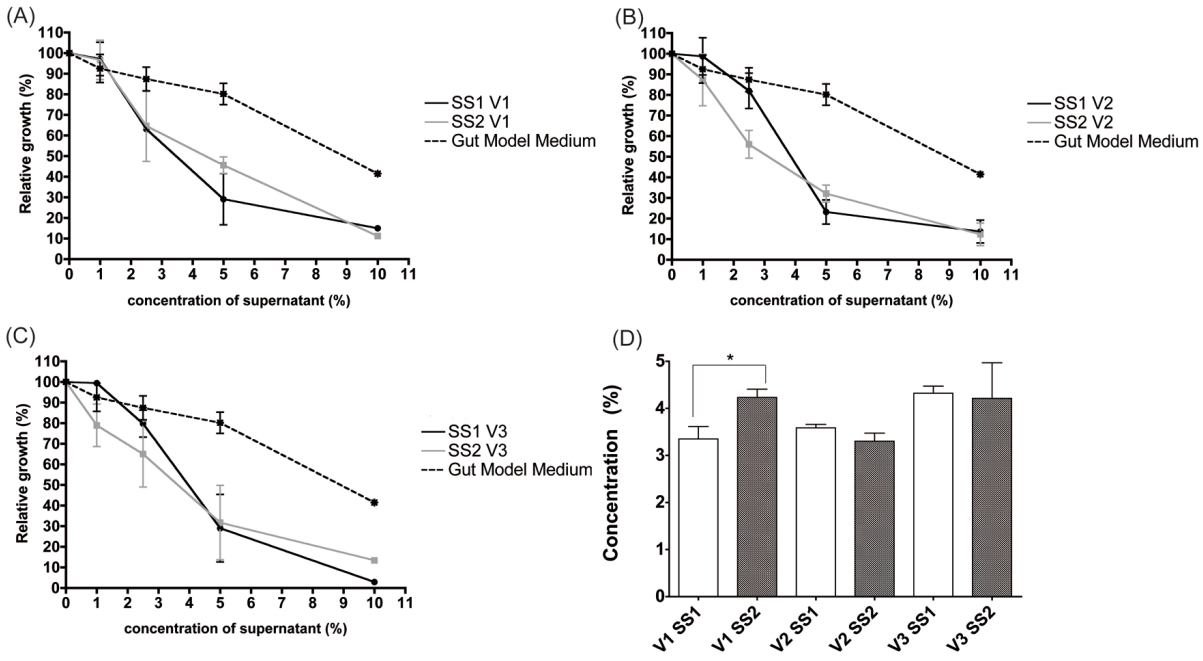


Figure 18. Cytotoxic effect of supernatants recovered from Vessel 1 (A), Vessel 2 (B), and Vessel 3 (C) of the colonic model system, before (SS1) and after (SS2) administration of *K. marxianus* B0399.

Cytotoxicity was assessed by co-incubating HT29 cells with increasing concentration (0%, 1%, 2.5%, 5%, 10%) of fermentation supernatants (filled circle: SS1, filled square: SS2, star: Gut Model Medium) followed by DAPI staining. Results are expressed as means of relative HT29 cell growths (%) of 2 colonic models ± SEM. For each colonic model, measurements were performed in triplicate. EC_{50} values were calculated from the growth curves shown in Figures 18A, B, and C for SS1 and SS2. Figure 18D shows the comparison of EC_{50} values in SS1 and SS2 for each vessel. $^{*}P < 0.05$

CHAPTER 3 - *IN VITRO* EVALUATION OF THE PROBIOTIC ACTIVITY OF *K. MARXIANUS* B0399

In recent years, an evolving number of studies suggested that the administration of probiotics plays a role in human health-promotion. In the present study, we assessed the probiotic potential of the food-grade yeast strain *K. marxianus* B0399, investigating a number of traits such as: i) adhesion to the intestinal epithelium; ii) modulation of the immune response; iii) impact on the composition and fermentation potential of the human colonic microbiota; iv) modulation of the cytotoxicity of the microbiota metabolites.

Using Caco-2 cells, a largely accepted *in vitro* model, we demonstrated that *K. marxianus* B0399 is a strongly adhesive strain. It is noteworthy that health-promoting effects of probiotic strains might be partly dependent on their persistence in the intestine and adhesion to mucosal surfaces (Collado et al., 2009).

A further important characteristic of potential probiotic candidates is the capacity to modulate the immune response of the host. In fact, a finely tuned balance between immune responses and tolerance to the gut microbiota is required at the edge of the colonic epithelium for preventing intestinal inflammation. Several *in vitro* and *in vivo* studies demonstrated two main effects of probiotics on the host immunity: i) strengthening the immunological barrier by stimulating the development and maintaining the state of alert of innate and adaptive immune system; ii) decreasing immune responsiveness to unbalanced inflammatory conditions.

Both these health-promoting activities are accomplished through an effective modulation of the balance of pro- and anti-inflammatory cytokines production (Vanderpool et al., 2008). Many probiotic species have been demonstrated to share a relatively common immune pattern, as a reduction in Th2 cytokines (i.e., IL-4, IL-5, IL-6, IL-10, IL-13) or a shift towards Th1-mediated immunity (i.e., IL-2, TNF-α, IFN-γ production). However, distinctive effects are often strain-specific and therefore the assessment of the immune potential of novel probiotics is a challenging research area in food microbiology (Collado et al., 2007; Delcenserie et al., 2008). Nowadays, very little is known about the immune potential of *Kluyveromyces* genus (Romanin et al., 2010). In the only study to date reported in literature, Romanin et al. (2010) demonstrated that probiotic *Kluyveromyces* spp. isolated from kefir counterbalanced the inflammatory action of flagellin, inducing a down-regulation of NF-kappaB signalling in epithelial cells *in vitro*, as well as expression of other pro-inflammatory chemokines such as CXCL8 and CXCL2.

In the present study, we evaluated the immunomodulatory potential of *K. marxianus* B0399 towards human PBMCs and Caco-2 cells. In PBMCs, *K. marxianus* B0399 induced the production of the pro-inflammatory cytokines IL-1β, TNF-α, IFN-γ and IL-6, which are known to play a crucial role in the host defence mechanism. A similar IL-6 and TNF-α overproduction was demonstrated in PBMCs exposed to well-established probiotic strains of lactobacilli, streptococci, *Leuconostoc* spp. and *B. breve* (Gaudana et al., 2010; Kekkonen et al., 2008; Timmerman et al., 2007). Notably, when *K. marxianus* B0399 was co-incubated with LPS, the concentration of TNF-α and IL-6 decreased to values similar to those detected in yeast-stimulated PBMCs without LPS. These data are in agreement with previous findings, which demonstrated that probiotic *L. rhamnosus* and *L. gasseri* strains were capable to diminish in different manner the release of TNF-α, IL-6 and IFN-γ in LPS-stimulated macrophage and PBMCs (Matsumoto et al., 2005; Pena et al., 2003). Interestingly, a similar

behaviour was also determined using the in vitro model system Caco-2 cells. In fact, in presence of a co-stimulation with *K. marxianus* B0399 and LPS, a significant decrease in concentration of the pro-inflammatory cytokines IFN-γ and IL-12 and chemokines IP-10 and IL-8 was demonstrated. In particular, a decreased production of IL-8 in response to inflammatory stimuli (LPS, TNF-α, IL-1β, enteropathogenic bacteria) was described for a wide array of probiotic bacteria (Candela et al., 2008; Frick et al., 2007; Kamada et al., 2008). Indeed, a massive and protracted IL-8 release by colonocytes, associated with enteropathogenic infections, leads to a persistent inflammation and epithelial barrier dysfunction (Roselli et al., 2006).

The ability of *K. marxianus* spp. to modulate the composition and the functional activity of the human intestinal ecosystem is poorly understood. In this perspective, we aimed at investigating how the *K. marxianus* B0399 administration impacts on the gut ecosystem, using a three-stage colonic model system that simulates the human colon.

Yeast administration did not mediate any significant modification in the total bacterial counts nor in the concentration of the predominant and subdominant bacterial groups. Notably, administration of *K. marxianus* B0399 provoked a significant increase in bacteria belonging to the health-promoting genus *Bifidobacterium* in the first and second stages of the colonic model system, which simulate the proximal and transverse colon. Whilst the metabolic potential of *K. marxianus* in the human gut has not been fully explored, it has been reported that this yeast can improve the growth and survival of bifidobacteria in complex food matrices (Rada, 1997). Indeed, it has been described that LAB growth can be stimulated by vitamins or amino acids produced by yeasts (Roostita and Fleet, 1996). Furthermore, it cannot be excluded that a small fraction of the *K. marxianus* B0399 added to the colonic model partially auto-lysates, releasing polysaccharides such as glucan and mannan, main constituents of the yeast cell wall. These polysaccharides can be converted into oligosaccharides, which are

known to stimulate the growth of *Bifidobacterium* spp. in the human and animal intestines (Belem and Lee, 1998)).

The administration of *K. marxianus* B0399 induced a significant increase of acetate and propionate over the course of the experiment. SCFAs, main end products of the carbohydrate fermentation, are demonstrated to play a pivotal role in the physiology and metabolism of the human colon. In particular, they provide energy for the intestinal colonocytes and promote epithelial cell growth (Jacobs et al., 2009). Even if the fermentation capability of *K. marxianus* to produce acetate has been described (Fonseca et al., 2007), the increase in concentration of acetate is consistent with the yeast-mediated modification in composition of the colonic microbiota, since *Bifidobacterium* spp. are principal producers of acetate (Jacobs et al., 2009). The relevant increase of acetate concentration in the colonic model system represents a valuable endpoint of the probiotic supplementation, since decreased levels of acetate and propionate have been correlated with gut metabolic profiles of patients affected by a variety of functional gastrointestinal disorders (Huda-Faujan et al., 2010; Marchesi et al., 2007).

Finally, we demonstrated that the administration of *K. marxianus* B0399 modulated a decrease of the cytotoxic potential of the cultural supernatant from the first vessel of the colonic model system. Our results are in agreement with those reported in literature, which showed that alteration of gut microbiota related to probiotic consumption may alter various parameters of faecal water activity by reducing toxicity (Pearson et al., 2009). The aqueous phase of human faeces contains bioactive compounds likely to interact with colonic epithelium. They include potentially harmful components, such as bile acids, fatty acids, N-nitroso compounds and heterocyclic amines, as well as compounds that are potentially beneficial, such as polyphenols and SCFAs (Pearson et al., 2009). Indeed, cytotoxicity of faecal water is reported to be a risk biomarker, since several studies correlated toxicity of this

faecal fraction with a higher colonic cell proliferation and increased colon cancer risk (Glinghammar et al., 1997).

In conclusion, the effects of *K. marxianus* B0399 on adhesion, immune function and colonic microbiota demonstrate that this strain possesses a number of beneficial and strain-specific properties desirable in a microorganism considered for application as a probiotics.

CHAPTER 4 - IRRITABLE BOWEL SYNDROME AND PROBIOTICS: AN *IN VIVO* STUDY

In physiological conditions, despite conservation at the highest taxonomic ranks, the intestinal microbiota is markedly individual-specific at species level, and a host-driven "top-down" assembly of the symbiotic microbial community has been suggested (Benson et al., 2010). Recently, it has been hypothesised that high taxonomic level unbalances of the human gut microbiota can be responsible for important modifications of the host physiological status, being associated with a number of gastrointestinal disorders (Mazmanian et al., 2008; Turnbaugh et al., 2009; Maccaferri et al., 2011). Irritable Bowel Syndrome (IBS) is the prevalent functional GIT disorder with a worldwide prevalence of 10-20% (Longstreth et al., 2006). IBS sufferers can be grouped into three main symptom subtypes: diarrhoea-predominant IBS (D-IBS), constipation-predominant IBS (C-IBS) and mixed bowel habit IBS (M-IBS). The cause of the disease is thought to be multifactorial and may include dysmotility, abnormal gut sensation, genetic, microbial and dietary factors, as well as low-grade inflammation (Codling et al., 2009). Several studies using qPCR, FISH and sequencing of 16S rDNA libraries reported an intestinal dysbiosis in patients suffering from IBS, in terms of specific compositional changes associated with the disorder (Malinen et al., 2005; Kassinen et al. 2007; Kerckhoffs et al., 2009; Tana et al., 2010; Maukonen et al., 2006; Lyra et al., 2009). However, in most of these studies, the overall microbiota was not covered, as the quantified bacteria were predetermined according to primer or probe sequences.

Only very recently, Rajilić-Stojanović et al. (2011) performed an in depth analysis of the human intestinal microbiota in IBS using a phylogenetic microarray targeting the bacterial 16S rRNA gene, demonstrating a significant decrease of *Bacteroidetes* (mainly belonging to *Bacteroides* and *Prevotella*), bifidobacteria and *Faecalibacterium prausnitzii*, and an increase in *Firmicutes*.

In the present study, we aimed at characterising the intestinal microbiota of 19 subjects suffering from diagnosed IBS (10 D-IBS, 5 M-IBS, 4 C-IBS), who were enrolled in a monocentric trial for evaluating the efficacy of a novel probiotic yoghurt (Lisotti et al., 2011) Indeed, different studies have suggested how probiotics may alleviate IBS symptoms and several mechanisms of action have been proposed (Marteau, 2010; Spiller, 2008). The novel dairy probiotic preparation investigated in the present study contained *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* and was supplemented with *B. animalis* subsp. *lactis* Bb12, which has been described as part of a probiotic formulation useful to manage IBS (Simrén et al., 2010; Søndergaard et al., 2011) and *K. marxianus* B0399, a novel probiotic lactic yeast we have been recently characterising for its potentially beneficial properties (Maccaferri et al., 2012).

A fully validated High Taxonomic Fingerprint Microbiota Array (HTF-Microbi.Array; Candela et al., 2010) was used to characterise the intestinal microbiota of the IBS subjects. This DNA microarray, based on the LDR technology (Castiglioni et al., 2004), is a highly specific, reproducible and sensitive tool that enables specific detection and approximated relative quantification of 16S rRNAs from 30 phylogenetically related groups, which cover about the 95% of the human intestinal microbiota (Rajilić-Stojanović et al., 2007). Differently from other DNA microarray platforms already reported in literature, the HTF-Microbi.Array is specifically designed to monitor the high level taxonomic unbalances of the core functional microbiome that could have an impact on the host physiological status (Mazmanian et al.,

2008; Turnbaugh et al., 2008; Hamady and Knight, 2009). Conversely, it remains blind to the species-level inter-individual variability. In the perspective of assessing the most relevant unbalances characterising the IBS microbiota, we compared compositional data from the faecal microbiota of IBS subjects obtained before the probiotic intake against a cohort of 24 healthy adults, comparable for sex and age, deriving from previous descriptive studies (Candela et al., 2010; Candela et al., 2011, personal communication). Finally, since the dairy probiotic product tested in this study has been demonstrated to improve bloating, bowel movement abnormality, as well as reduce abdominal pain in IBS patients (Lisotti et al., 2011), we assessed if the IBS-associated unbalances of the intestinal microbiota were reverted by the probiotic yoghurt.

Subjects and study design

The study group consisted of 19 subjects suffering from diagnosed IBS, who were enrolled in the intervention study. IBS patients (mean age = 33.6 ± 9.1) fulfilled the Rome III criteria for the diagnosis of IBS (Longstreth et al., 2006). Exclusion criteria included pregnancy or lactation, chronic intestinal disease (i.e., inflammatory bowel disease or coeliac disease) or severe systemic disorders, lactose intolerance or food allergies. Patients who in the 2 months prior to study entry had taken medication, such as antibiotics, corticosteroids or functional foods containing pre- or probiotics, were also excluded from the study. Each subject signed the informed consent prior to enter the study. The study protocol was conforming to the ethical guidelines of the "World Medical Association Declaration of Helsinki".

IBS patients were subjected to a 4-week study period, and were daily receiving a probiotic yoghurt containing *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and supplemented with *K. marxianus* B0399 and *B. animalis* subsp. *lactis* Bb12. The total daily amount of *K. marxianus* B0399 was 1-4 x 10^7 CFU, whilst the amount of *B. animalis* subsp. *lactis* Bb12 was 3-5 x 10^9 CFU. Consumption of other probiotics was not allowed during the intervention. All subjects were advised to follow their usual dietary habits and not to undertake any medication.

For the comparative analysis of the IBS microbiota composition before the probiotic intervention, a cohort of 24 healthy subjects comparable for age and sex and already characterised for their intestinal microbiota profiling was considered.

These compositional data were retrieved using the same analytical approach undertaken in this study (Candela et al., 2010; Candela et al., 2011, personal communication).

Faecal samples

During the study, two faecal samples were collected from each subject suffering from IBS. Samples were taken before beginning the probiotic supplementation for assessing the individual baseline, and after the 4-week supplementation. Samples were immediately stored in anaerobic containers and frozen within 4 h to -70°C until analysis.

Extraction and purification of microbial DNA from faecal samples

Total microbial DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen) with a modified protocol, as previously described by Biagi et al (2010). Final DNA concentration was determined by using NanoDrop ND-1000 (NanoDrop Technologies).

Characterisation of the intestinal microbiota by HTF-Microbi.Array

The intestinal microbiota of the IBS subjects enrolled in the study was characterised using the fully validated DNA microarray HTF-Microbi.Array, which target 30 phylogenetically related groups. The analysis was performed at the baseline and after the 4-week probiotic supplementation. 16S rRNA was amplified using universal forward primer 16S27F and reverse primer r1492, following the protocol previously described (Candela et al., 2010). PCR products were purified by using a Wizard SV gel and PCR clean-up System purification kit (Promega Italia), according to the manufacturer's instructions, eluted in 20 µl of sterile water,

and quantified with the DNA 7500 LabChip Assay kit and BioAnalyzer 2100 (Agilent Technologies).

Ligase Detection Reaction and hybridisation of the products on the universal arrays were performed according to the protocol described by Castiglioni et al. (2004). except for the probe annealing temperature, set at 60 °C.

PCR-DGGE analysis of the faecal samples

Studies on the microbial DNA fingerprints derived from PCR-DGGE analysis were performed for the IBS subjects, at the baseline and following the 4-week probiotic supplementation. Amplification of the V2-V3 region of the bacterial 16S rRNA gene was carried out using the universal eubacterial primers GCclamp-HDA1 and HDA2, according to the protocol previously described by Maccaferri et al. (2012). DGGE gel images were analysed using the FPQuest Software Version 4.5 (Bio-Rad). In order to compensate for gel-to-gel differences and external distortion to electrophoresis, the DGGE patterns were aligned and normalized using an external reference ladder composed by known bacterial species. After normalization, bands were defined for each sample using the appropriate densitometric curves. Bands constituting less than 1% of the total band area were omitted from further analysis. Similarity between DGGE profiles was determined by calculating the Pearson correlation. Clustering of the sample profiles was done using the UPGMA algorithm. Additionally, a Shannon diversity index was calculated to investigate the structural diversity of the microbial community.

Culture-independent and -dependent detection of *K. marxianus* B0399 in the faecal microbiota

Dynamics of yeast population and detection of the administered yeast during the study were assessed by PCR-DGGE and selective plate counting, respectively. Approximately 250 nucleotides of the 5'-end region of the 26S rRNA gene were amplified by PCR using the yeast-universal primer set GC-clamped NL1 and LS2, according to Cocolin et al. (2000). The PCR-DGGE experimental protocol was slightly modified by performing annealing at 56 °C for 25 s and extension at 72 °C for 30 s, in order to prevent cross-amplification of bacterial DNA. Band identity was confirmed by comparison of the positions in the gel length with those of reference yeast DNA.

Detection of the survival and quantification of the growth of *K. marxianus* B0399 along the intervention were performed by plate counting at 37°C in lactic-yeast selective MV2 agar. 1:5 (w/w) faecal dilutions in anaerobic PBS [0.1 M PBS (pH 7.4)] were prepared and the samples were homogenised in a stomacher (Seward Ltd.) for 2 min. After homogenisation, faecal samples were serially diluted using 10-fold serial dilutions down to a final dilution of 10^{-5}. All plating counts were performed in triplicate.

Statistics

All arrays were scanned with ScanArray 5000 scanner (Perkin Elmer Life Sciences), at 10 μm resolution. Fluorescent images were obtained with different acquisition parameters for both laser power and photo-multiplier gain, in order to avoid saturation. Fluorescence intensities were quantitated by ScanArray Express 3.0 software, using the "Adaptive circle" option, letting diameters vary from 60 to 300 μm. To assess whether a probe pair was significantly above the background (i.e. was present or not), one-sided t-test was performed. Nonparametric Kruskal-Wallis test was used to determine the statistical differences among the

IBS subtypes and/or treatment conditions. Mann-Whitney U-test was used for pairwise comparisons. A $P < 0.05$ was considered statistically significant. Hierarchical clustering of HTF-Microbi.Array profiles was carried out using the statistical software R (http://www.r-project.org). The Euclidean distance among sample profiles was calculated and Ward's method was used for agglomeration. Redundancy analysis and RDA ordination diagram were performed using CANOCO for Windows 4.02 and CanoDraw 3.10 (Microcomputer Power), respectively. Monte Carlo permutation test was done at 199 random permutations, in order to assess significant differences.

Bacterial counts and DGGE parameters were analysed by one-way ANOVA, using Tukey's post-test analysis when the overall P value of the experiment was below the value of significance ($P < 0.05$). An additional paired t-test was applied in order to assess the significance of the results of single pairs of data. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software).

Characterisation of the intestinal microbiota in IBS subjects

High taxonomic fingerprints of the faecal microbiota of the IBS subjects were depicted by HTF-Microbi.Array (Annex 3), and compared with those of healthy subjects deriving from previous descriptive studies (Candela et al., 2010; Candela et al., 2011, personal communication).

The main bacterial groups of the IBS microbiota were *Clostridium* cluster IV and XIV (25% and 21% of the total microbiota, respectively), followed by *Bacteroides*/ *Prevotella* (9.1%). Other subdominant bacterial groups found at relevant concentration in the IBS microbiota were lactic acid bacteria (7.8%, summing the hits of *Lactobacillaceae* family and those of the *Lactobacillus* species targeted by the HTF-Microbi.Array), as well as *Veillonella* genus (5.7%), *Bacilli* class (2.9%) and *Bifidobacteriaceae* family (1.2%).

Multivariate redundancy analysis of the relative abundance of targeted bacterial groups/species highlighted that the microbiota of IBS subjects is significantly different from that of healthy individuals ($P < 0.05$). Triplot of the RDA of the composition of the faecal microbiota of healthy and IBS subjects demonstrated that samples clearly separated on the basis of the health status (Figure 19). Furthermore, a clear separation of M-IBS and C-IBS, with respect to D-IBS was demonstrated.

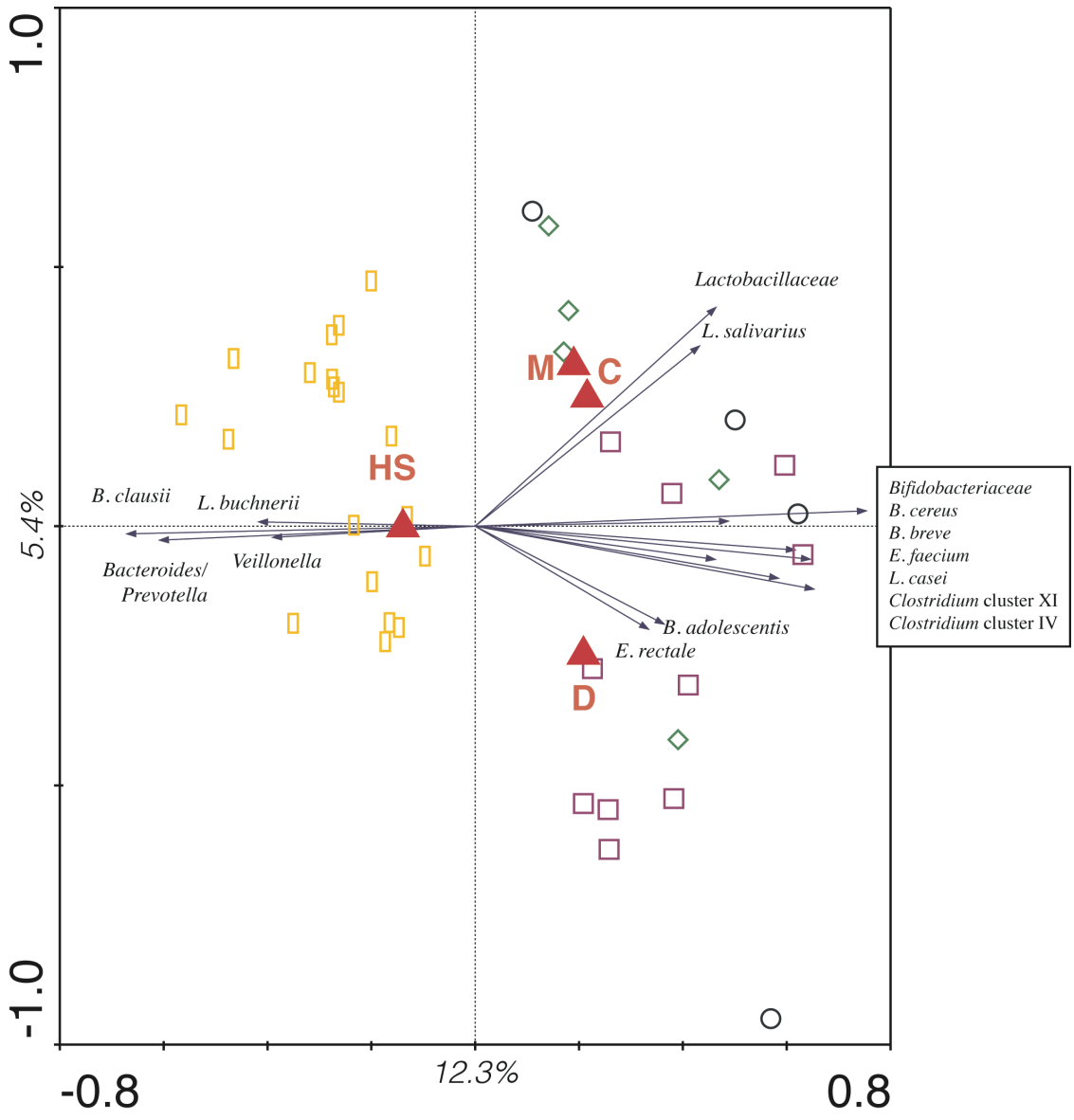


Figure 19. Triplot of the RDA of the microbiota composition of subjects suffering from IBS and healthy individuals. Healthy subjects (HS), M-IBS patients (M), C-IBS patients (C) and D-IBS patients (D) are indicated by yellow rectangles, green diamonds, black circles and purple square, respectively. Constrained explanatory variables (HS, M, C, D) are indicated by filled red triangles. Black arrows indicate responding bacterial subgroups that explain more than 15% of the variability of the samples. First and second ordination axes are plotted, showing 12.3% and 5.4% of the total variability in the dataset, respectively.

Table 3, 4 and Figure 20 (A, B, and C) show the bacterial groups significantly ($P < 0.05$) altered in IBS subjects with respect to healthy controls. In particular, the faecal microbiota of IBS subjects was demonstrated to be enriched in bacilli, *Bifidobacteriaceae*, *Clostridium* cluster IX, *E. rectale* and *Lactobacillaceae*. Notably, members of *Enterobacteriaceae*, *E. faecium*, *C. difficile* and *Campylobacter* spp. were also demonstrated to be enriched in the IBS microbiota, with respect to the faecal microbiota of healthy subjects. Conversely, the IBS microbiota was depleted in concentration of *Bacteroides*/*Prevotella* group and *Veillonella* genus.

A number of significant variations in specific phylogenetic groups have been demonstrated not only between IBS and healthy controls, but also among the different IBS subtypes or among each IBS subtype and healthy individuals (Table 3 and 4). Interestingly, C-IBS was different from D-IBS, with a significant depletion in *E. rectale* concentration (P = 0.024). On the other hand, M-IBS was different from D-IBS for a significant increase in *L. buchnerii* (P = 0.045).

	% of the total targeted microbiota				
Target phylogenetic group	**IBS**	**C-IBS**	**D-IBS**	**M-IBS**	**HS**
Bacillus cereus	1.04	0.9	1.04	1.17	0.29
Bacillus clausii	1.88	2.03	1.89	1.74	0.21
Bacteroides/Prevotella group	9.11	7.38	9.39	9.91	19.79
Bifidobacteriaceae	1.17	1.08	1.23	1.11	0.42
Bifidobacterium adolescentis	0.88	0.68	1.05	0.69	0.5
Bifidobacterium bifidum	0.68	0.62	0.72	0.67	0.47
Bifidobacterium breve	0.7	0.68	0.7	0.72	0.38
Bifidobacterium longum	0.8	0.79	0.83	0.76	0.41
Campylobacter spp.	0.64	0.65	0.61	0.7	0.41
Clostridium difficile	0.64	0.63	0.65	0.6	0.34
Clostridium cluster IX	1.1	1.14	1.16	0.96	0.54
Enterobacteriaceae	1.07	0.72	1.34	0.82	0.4
Enterococcus faecium	0.87	0.67	0.94	0.9	0.47
Eubacterium rectale	6.51	3.81	7.13	7.43	3.71
Lactobacillaceae	2.49	3.05	2.46	2.1	0.82
Lactobacillus casei	2.09	2.77	2.02	1.69	3.78
Lactobacillus salivarius	0.71	0.66	0.77	0.64	0.29
Lactobacillus buchnerii	1.56	1.37	1.1	2.62	0.69
Veillonella spp.	5.66	5.83	5.85	5.13	13.12

Table 3. Bacterial groups significantly altered in IBS subjects (IBS; Constipation IBS, C-IBS; Diarrhoea IBS, D-IBS; Mixed IBS, M-IBS), with respect to healthy subjects (HS). Data are expressed as % of the total microbiota targeted by HTF-Microbi.Array.

	***P* value of comparison among the different study groups**						
Target phylogenetic group	**IBS vs. HS**	**C-IBS vs. D-IBS**	**C-IBS vs. M-IBS**	**D-IBS vs. M-IBS**	**C-IBS vs. HS**	**D-IBS vs. HS**	**M-IBS vs. HS**
Bacillus cereus	**4.00E-08**	0.54	0.73	0.95	**0.013**	**8.50E-09**	**0.016**
Bacillus clausii	**5.20E-09**	0.95	0.9	0.59	**3.90E-04**	**1.60E-05**	**5.90E-05**
Bacteroides/Prevotella group	**1.20E-05**	0.64	0.56	0.77	**0.0021**	**0.001**	**0.016**
Bifidobacteriaceae	**2.30E-08**	0.95	0.73	1	**0.0015**	**2.10E-05**	**2.40E-04**
Bifidobacterium adolescentis	**0.0013**	0.45	0.73	0.44	0.25	**0.0016**	0.12
Bifidobacterium bifidum	**0.0039**	0.73	0.56	0.77	0.3	**0.0055**	0.14
Bifidobacterium breve	**5.40E-04**	0.95	0.9	0.59	0.17	**0.0012**	0.074
Bifidobacterium longum	**3.80E-05**	0.95	1	0.77	0.094	**8.70E-05**	**0.039**
Campylobacter spp.	**9.30E-04**	0.95	0.9	0.44	0.11	**0.037**	**0.0021**
Clostridium difficile	**3.10E-04**	1	0.9	0.86	0.13	**0.001**	0.051
Clostridium cluster IX	**5.30E-06**	0.73	0.73	0.95	**3.90E-03**	**0.0012**	**0.0027**
Enterobacteriaceae	**2.00E-06**	0.95	0.73	0.77	0.062	**7.20E-05**	**9.50E-04**
Enterococcus faecium	**1.30E-06**	0.3	0.19	0.95	0.21	**8.00E-06**	**3.50E-04**
Eubacterium rectale	**1.70E-05**	**0.024**	0.19	0.77	0.13	**8.70E-05**	**0.0079**
Lactobacillaceae	**1.70E-05**	0.64	0.9	0.59	**0.024**	**0.001**	**0.0043**
Lactobacillus casei	**0.0046**	0.45	0.29	0.86	0.53	**0.013**	**0.03**
Lactobacillus salivarius	**5.30E-05**	0.84	0.73	0.95	0.062	**4.90E-04**	**0.022**
Lactobacillus buchnerii	**2.80E-04**	0.64	0.19	**0.045**	0.21	**0.013**	**1.60E-04**
Veillonella spp.	**0.0011**	0.95	0.9	0.95	0.13	**9.50E-03**	**0.03**

Table 4. *P* values of the modifications among bacterial groups significantly altered in IBS subjects (IBS; Constipation IBS, C-IBS; Diarrhoea IBS, D-IBS; Mixed IBS, M-IBS), with respect to healthy subjects (HS). Significance variations ($P < 0.05$) are visualized in bold.

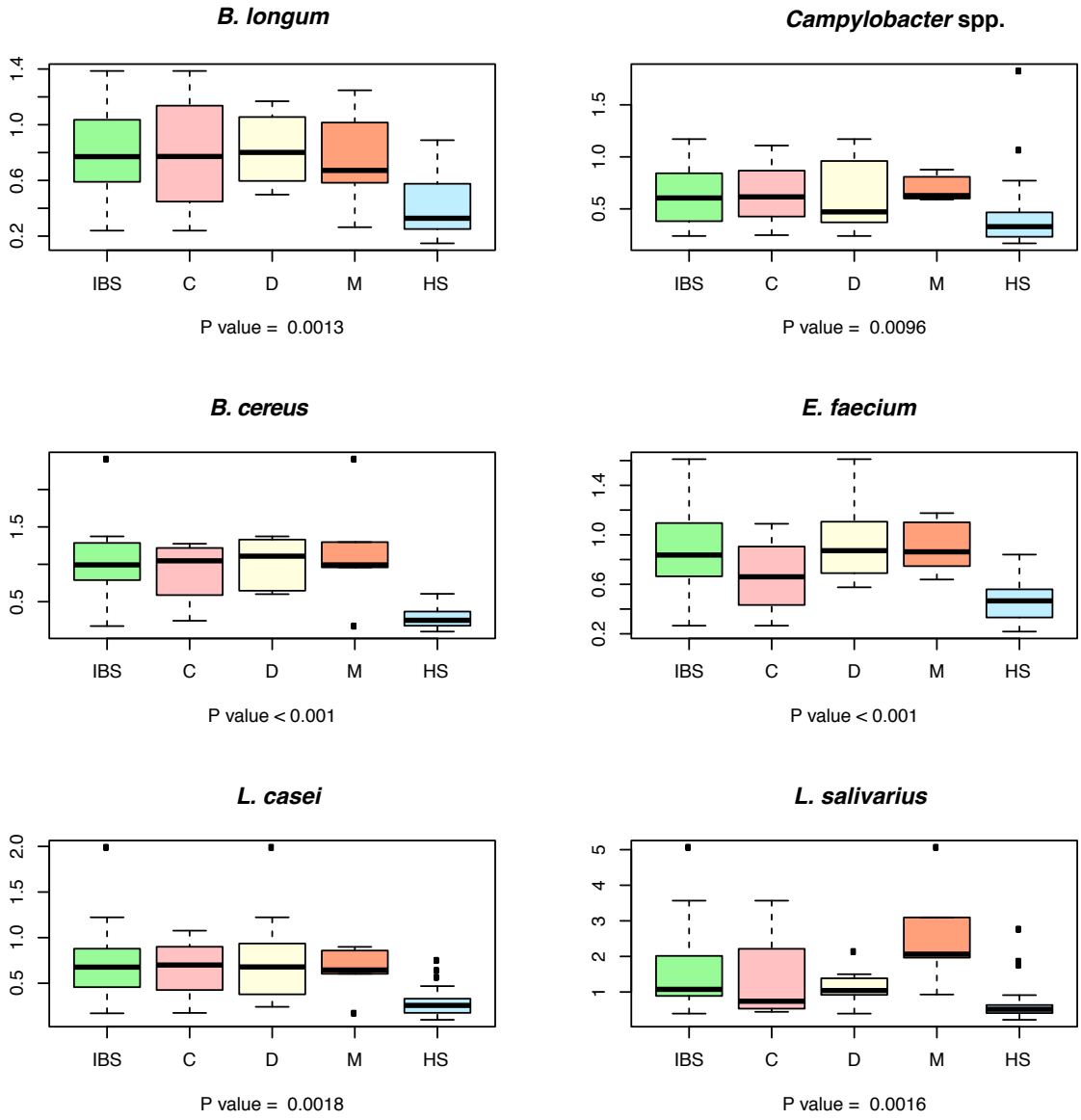


Figure 20 (A). Box plots of the HTF-Microbi.Array relative abundance percentages corresponding to probes which showed an overall significantly different response between IBS, C-IBS (C), D-IBS (D), M-IBS (M) and healthy subjects (HS). ($P < 0.05$)

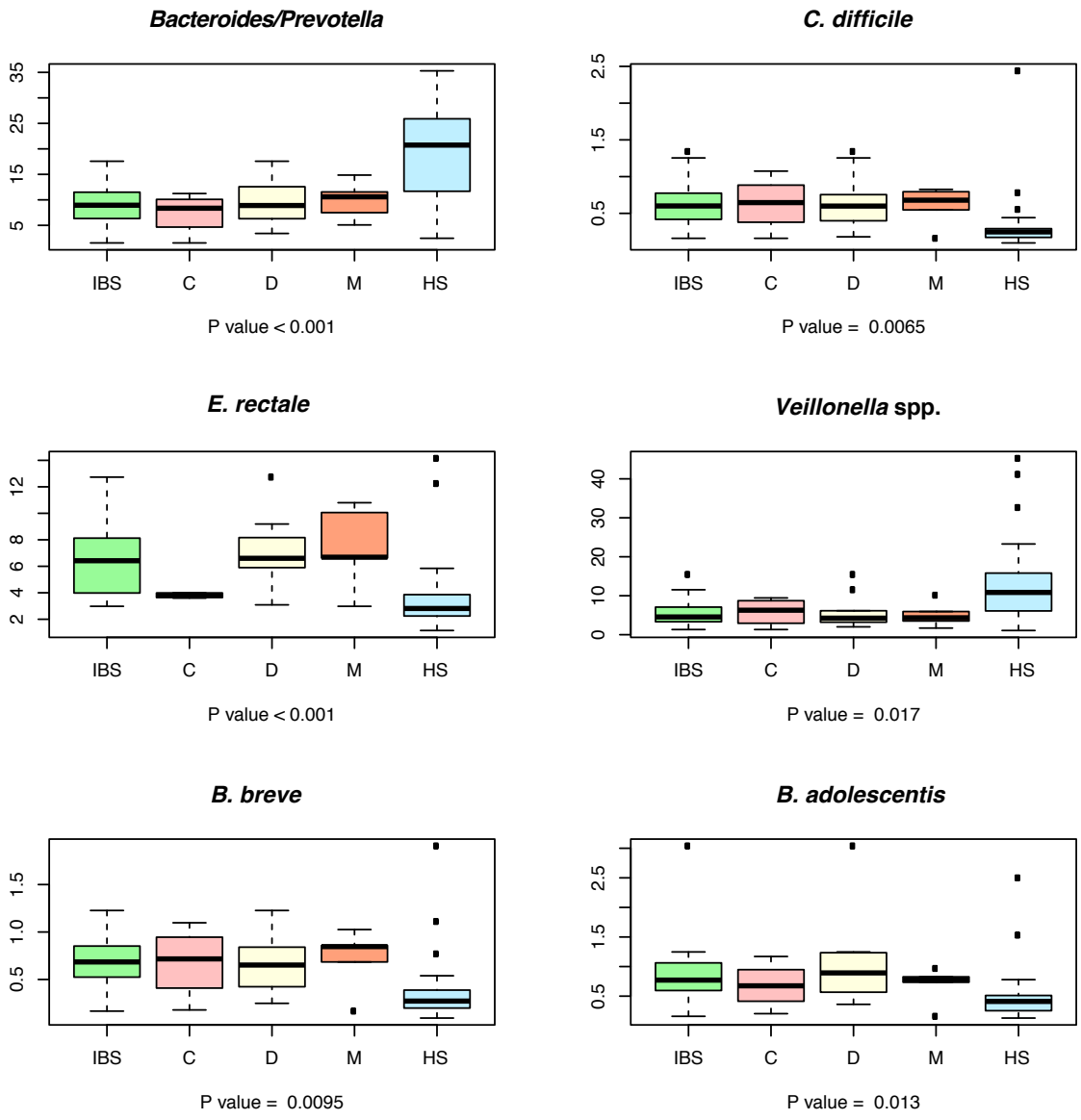


Figure 20 (B). Box plots of the HTF-Microbi.Array relative abundance percentages corresponding to probes which showed an overall significantly different response between IBS, C-IBS (C), D-IBS (D), M-IBS (M) and healthy subjects (HS). ($P < 0.05$)

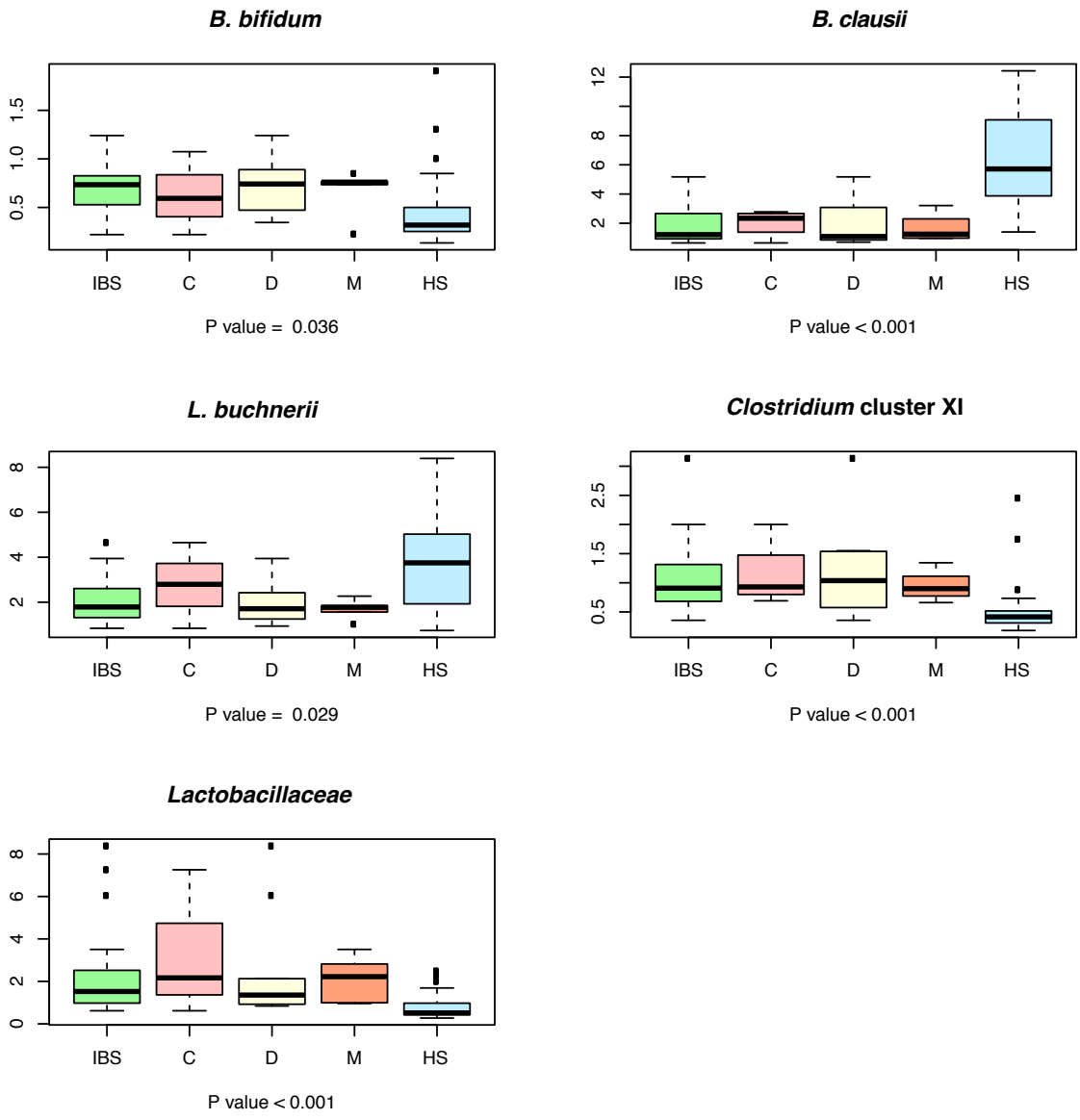


Figure 20 (C). Box plots of the HTF-Microbi.Array relative abundance percentages corresponding to probes which showed an overall significantly different response between IBS, C-IBS (C), D-IBS (D), M-IBS (M) and healthy subjects (HS). ($P < 0.05$)

Furthermore, the comparison of the IBS microbiota composition before and after intake of the probiotic yoghurt supplemented with *B. animalis* subsp. *lactis* Bb12 and *K. marxianus* B0399 was performed. The microarray datasets of the faecal microbiota of the IBS subjects analysed in the present study were hierarchically clustered on the basis of the signal intensity of the HTF-Microbi.Array oligonucleotide probes (Figure 21). According to the main phylogenetic features of the faecal microbiota, two groupings were assessed. A marked inter-individual diversity was demonstrated, since the majority of the samples before and after intervention clustered together, and no grouping according to the probiotic intervention was depicted.

Moreover, PCR-DGGE analysis was used to retrieve an additional picture on the dynamics of the bacterial community before and after intervention. PCR-DGGE confirmed that the biodiversity of the intestinal microbiota was not influenced by the probiotic treatment, as assessed by the richness and Shannon indices ($P > 0.05$). Mean values of the richness index ranged from 17.5 (T0) to 19.6 (T1), whereas mean values of the Shannon index ranged from 2.75 (T0) to 3.02 (T1). Finally, the peak heights of DGGE densitometric curves were analysed using the Mann-Whitney U-test, in order to assess if the most relevant single-species abundances were affected by the probiotic administration. No significant changes in species abundance were found when comparing T0 and T1.

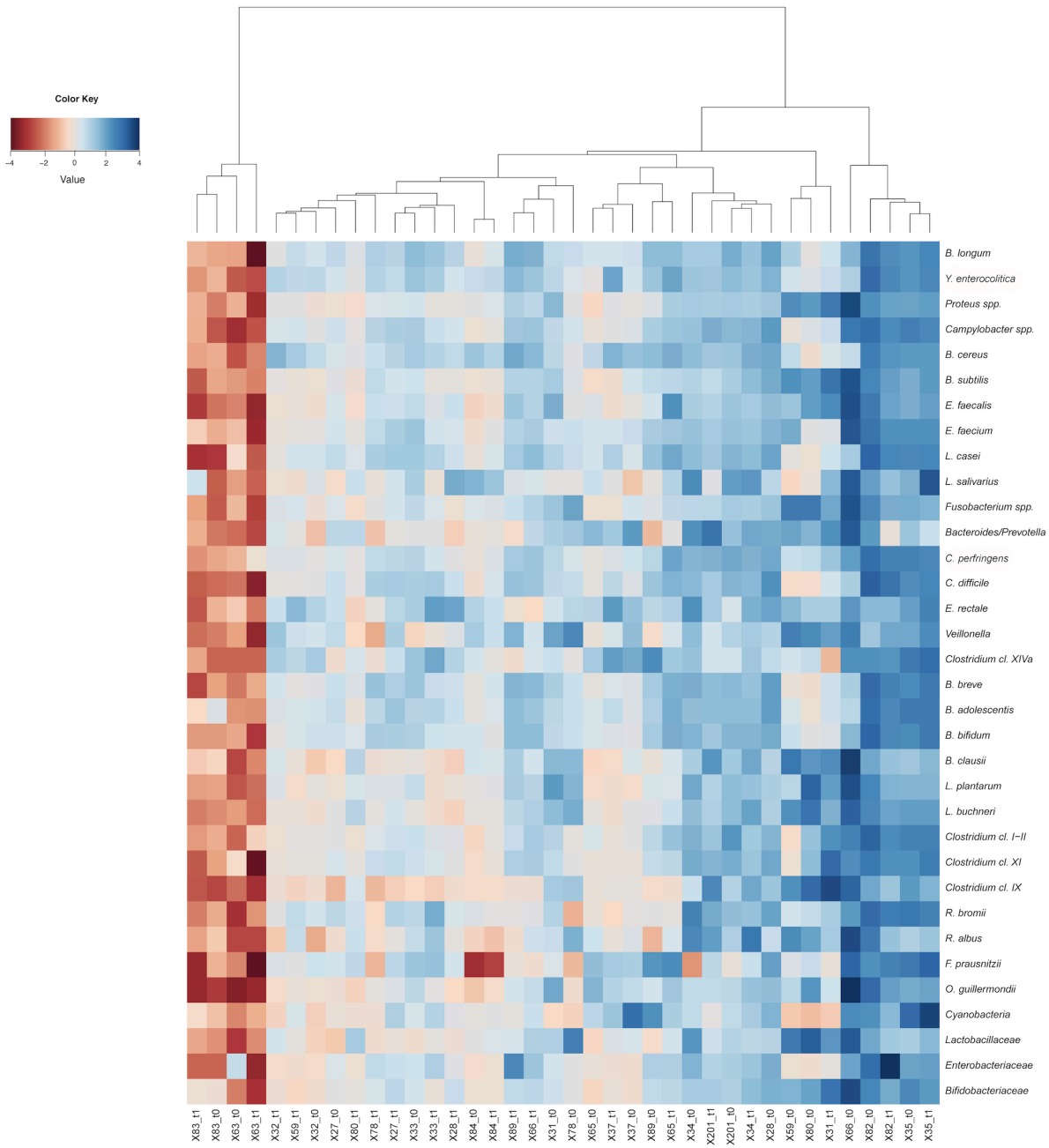


Figure 21. Hierarchical clustering of the HTF-Microbi.Array profiles of IBS subjects before and after the probiotic administration. Microarray fingerprints at the baseline are indicated by t0, whereas fingerprints after the probiotic intervention are indicated by t1. Colour intensity represents the relative bacterial abundance in the sample, in relation to the study population. Euclidean distance and Ward's clustering method were applied to log-transformed data.

Evaluation of the survival of *K. marxianus* B0399 along the probiotic intervention

The survival of *K. marxianus* B0399 along the probiotic intervention was tested using selective plate counting for lactic yeasts and semi-quantitative PCR-DGGE analysis followed by band identification.

Total count of faecal lactic yeasts showed negligible levels (< 100 CFU/g of faeces) at T0 in 15/19 subjects (75%), whilst the remaining 4 subjects had a basal concentration of (3.2 ± 0.6) x 10^3 CFU/g. Following the probiotic treatment, 16/19 subjects (84% of the study population, $P < 0.001$) were positive for yeast colonisation, which reached a T1 concentration of (4.3 ± 1.2) x 10^5 CFU/g. The presence of *K. marxianus* within the micro-eukaryotic faecal microbiota was confirmed by PCR-DGGE. PCR-DGGE analysis, whose sensitivity (~10^5 yeast cell/mL) was not sufficient to detect *K. marxianus* at T0, confirmed the presence of a clear band corresponding to *K. marxianus* (99% sequence identity with *K. marxianus* 13MCHS 26S ribosomal RNA gene, Annex 2) at T1 in 14/19 subject (74% of the study population, $P < 0.001$).

CHAPTER 4 - IRRITABLE BOWEL SYNDROME AND PROBIOTICS: AN *IN VIVO* STUDY

Recently, a number of studies investigated the unbalances that characterise the intestinal microbiota of patients suffering from IBS (Codling et al., 2009: Malinen et al., 2005; Kassinen et al. 2007; Kerckhoffs et al., 2009; Tana et al., 2010; Maukonen et al., 2006; Lyra et al., 2009). However, the rationale beyond differences in the microbiota composition between IBS patients and healthy individuals is still evolving. Furthermore, a growing number of studies have evaluated the response of IBS to probiotics, and few recent systematic reviews and meta-analyses suggested that probiotics appear to be, to varying extent, effective or at least promising in the amelioration of the well-being status of IBS subjects (Marteau, 2010; Spiller, 2008; Ringel and Ringel-Kulka, 2011).

In the present study, we analysed the faecal samples of 19 subjects suffering from IBS enrolled in a clinical trial for the evaluation of the efficacy of a new probiotic yoghurt containing *K. marxianus* B0399 and *B. animalis* subsp. *lactis* Bb12 (Lisotti et al., 2011). Firstly, we evaluated the shift in the microbiota composition of the IBS patients before the probiotic administration, by comparing their microbiota profiles with those of a cohort of 24 healthy subjects, matched for sex and age, and previously characterised (Candela et al., 2010; Candela et al., 2011, personal communication). Successively, we assessed the impact of the probiotic yoghurt on the gut microbiota composition.

We demonstrated that IBS microbiota is different from that of healthy individuals due to an unbalance in a number of commensal species, with an increase in relative abundance of lactobacilli, *B. cereus* and *B. clausii*, bifidobacteria, *Clostridium* cluster IX and *E. rectale*, and a decrease in abundance of *Bacteroides*/*Prevotella* group and *Veillonella* genus. Furthermore, we demonstrated that some bacterial groups of the human intestinal microbiota, recently defined as pathobionts, are increased in concentration in the IBS microbiota. The so-called pathobionts are bacteria that can asymptomatically colonise the human GIT, but possessing pro-inflammatory characteristics they might have a role in causing disease when, due to a dysbiosis, they increase in concentration (Cerf-Bensussan and Gaboriau-Routhiau, 2010). In the present study, members of the *Enterobacteriaceae* family, *E. faecium*, *C. difficile* and *Campylobacter* spp. were demonstrated to be enriched in the IBS microbiota, with respect to the faecal microbiota of healthy subjects.

That the intestinal microbiota of subjects suffering from IBS deviates from the definition of a standard core microbiota in healthy conditions is a matter of fact, since an increasing number of studies evidenced peculiar modifications in the composition of the human intestinal microbial ecosystem correlated to health and disease status. However, the definitions of both IBS microbiota and standard core microbiota are challenged by the technological advancement, which is giving novel insights in understanding the dynamic interplay of the microbial species thriving human gut. To date, a limited number of phylum- and group-level differences have been demonstrated comparing IBS patients to healthy subjects, whilst several alterations in abundance at genus and species level have been identified, leading to results that are sometimes controversial (Salonen et al., 2010).

In particular, our results are in accordance with those demonstrating an increase in abundance of *Lactobacillus* genus in IBS (Kassinen et al., 2007; Tana et al., 2010), which has been associated with augmented concentration of the organic acids propionate and acetate, that in

turn were correlated with abdominal pain, bloating and anxiety by Tana et al. (2010). Conversely, other studies indicated a depletion of lactobacilli as a characteristic of the IBS microbiota (Malinen et al., 2005; Kerchkoffs et al., 2009). Interestingly, a recent study performed using a DNA phylogenetic microarray by Rajilić-Stojanović et al. (2011) demonstrated a trend similar to that reported in this study, in relation to the dynamics of the *Bacteroides*/*Prevotella* group and bacilli, with the first group depleted in subjects suffering from IBS and the second one increased in abundance in the IBS microbiota. We also demonstrated that the IBS microbiota showed enrichment in bifidobacterial concentration, result which is in contrast with previous findings reporting decreased bifidobacterial concentrations in IBS patients (Kerckhoffs et al., 2009; Rajilić-Stojanović et al., 2011). The HTF-Microbi.Array used in this study targets the entire *Bifidobacteriaceae* family and the *B. longum*, *B. adolescentis*, *B. breve* and *B. bifidum* species.

Numerous human studies and clinical trials have investigated the therapeutic benefit of probiotics in alleviating the symptoms of IBS, with a wide range of formulations and microbial species tested. Commonly used probiotic strains belong to *Bifidobacterium* or *Lactobacillus* genera, whilst less frequently used are strains of *Propionibacterium freudenreichii*, bacilli or yeasts (Wassenaar and Klein, 2008). Recently, we demonstrated in an in vitro study that *K. marxianus* B0399 possesses a number of beneficial properties, i.e. modulation of the immune response of PBMC and Caco-2 cells, impact on the metabolic activity of the intestinal microbiota and survival to simulated gastrointestinal environment, supporting its application as a probiotic (Maccaferri et al., 2012). The efficacy of a probiotic yoghurt including *K. marxianus* B0399 and *B. animalis* subsp. *lactis* Bb12 in the management of IBS has been investigated in an in vivo study (Lisotti et al., 2011). The authors showed that these probiotics provoked an improvement in abdominal pain, bloating and bowel movement abnormality.

We characterised the intestinal microbiota of the 19 IBS patients enrolled in the above mentioned clinical study, with the aim of evaluating the impact of the probiotic administration on the IBS-associated unbalances of the intestinal microbiota.

Using both HTF-Microbi.Array and PCR-DGGE, we demonstrated that the supplementation of *K. marxianus* B0399 and *B. animalis* subsp. *lactis* Bb12 for 4 weeks did not modulate the composition of the microbiota in the IBS patients. Indeed, a marked inter-individual diversity was evident, since the majority of the samples before and after intervention clustered together, and no groupings according to the probiotic intervention were depicted. Similarly, Shannon and richness indices of DGGE gels were not modified by the 4-week probiotic administration. At the light of the most recent findings, our results are in agreement with an increasing number of studies demonstrating that probiotic administration is often not accompanied by compositional modulations of the intestinal microbiota in subjects suffering from IBS (Michail and Kenche, 2011), microbiota-mediated systemic disorders (Larsen et al., 2011) and in healthy conditions (Vitali et al., 2010).

In conclusion, we improved the knowledge about the peculiar modifications characterising the intestinal microbiota of subjects suffering from IBS. Furthermore, we demonstrated that the beneficial effects of the probiotic yoghurt containing *K. marxianus* B0399 and *B. animalis* subsp. *lactis* Bb12 are not associated to significant modifications of the human intestinal microbiota. These results open a new scenario about the necessity of characterising the mechanism of action of clinically relevant probiotic strains not only towards the composition of the gut microbiota, but also taking into account its functionality.

POTENTIAL PREBIOTIC FLOURS FROM NATURAL SOURCES: IMPACT ON THE HUMAN GUT MICROBIOTA AND METABOLOME

CHAPTER 5 - *IN VITRO* FERMENTATION OF NOVEL DIETARY FIBRES

The gut microbiota is a complex and dynamic ecosystem that constantly interacts with the human metabolism, endowing the host with physiological traits that have not evolved in the host (Gill et al., 2006; Neish et al., 2009; Hooper et al., 2002). In particular, the intestinal microbiota can be regarded as a virtual organ able to exert a key contribution to the human energy balance. Thus, the host can be considered a meta-organism, whose metabolism results from the both the human and the collective microbial community counterparts (Kau et al., 2011). Diet is considered a major driver for changes in the compositional and functional relationship between microbiota and the host. In fact, dietary components are susceptible for metabolism by the intestinal microbial ecosystem, particularly influencing the growth and the metabolic activity of the dynamic bacterial populations thriving in the human colon (Laparra and Sanz, 2010).

To date, dietary fibres and prebiotics represent a useful dietary approach for influencing the composition of the human gut microbial community, since they are not completely metabolised by the digestive enzymes in the human small intestine. In particular, prebiotics are non-digestible food ingredients which are fermented by the gut microbiota and beneficially affect the host stimulating growth and/or activity of specific intestinal bacteria (Gibson et al., 2004). Most common prebiotics are non-digestible GOS and FOS, such as inulin and oligofructose. Inulin-type fructans are present in a range of different plants including wheat, onion, banana, garlic, leek and agave (Candela et al., 2010).

The biological effect of prebiotics mainly depends on their influence on the gut microbiota composition and metabolism, exerted through a number of different functional properties, such as prevention of pathogen adhesion and colonization, modulation of bowel habits, regulation of lipid and glucose metabolism and influence of the intestinal metabolome (Candela et al., 2010; Sherman et al., 2009). Several dietary fibers, including non-starch polysaccharides, whole-grain, cellulose, dextrans, chitins, pectins, β-glucans and waxes have been reported to potentially provide similar beneficial effects as those of inulin-type fructans (Laparra and Sanz, 2010; Costabile et al., 2008; Napolitano et al., 2009). Very recently, it has been demonstrated that prebiotic-enriched pasta could be a useful dietary tool to manipulate gut microbiome-mediated well-being endpoint (Russo et al., 2010; Russo et al., 2011).

BOX N. 5 - *METABOLOMICS AND DIET: FEEDING THE KNOWLEDGE*

Whereas the inter-individual diversity in composition of the human gut microbiota is large and commonly accepted, differences in the colonic metabolome has been found to originate mainly from variable metabolite concentrations, rather than from individual differences in the diverse composition of the metabolome. To date, ^{1}H-NMR, GC-MS and LC-MS methods have been set up in order to monitor changes in gut metabolome in response to exogenous stimuli.

SCFAs and organic acids are the main microbial metabolites detected in the faecal metabolome, using targeted GC-MS. Conversely, untargeted NMR-based metabolite profiling is a rapid, comprehensive technique which allow to detect a variety of different fermentation products in faeces, which include SCFAs, organic acids (i.e., succinate, pyruvate, fumarate, lactate), amino acids, uracil, trimethylamine, ethanol, glycerol, glucose, phenolic acids, cholate and lipid components.

Recently, NMR-based metabolomics platforms have been developed to study the impact of non-digestible food ingredients in the colonic metabolome. Metabolomic profiling might reveal new affected metabolic pathways useful in discovering potential biomarkers of metabolic activity of dietary fibres, prebiotics, or probiotics.

In the present study, we aimed at investigating the impact of four flours characterised by a naturally high content in dietary fibres [Wholegrain rye (WGR), Nutriwheat (wholegrain wheat, NW), Pulses (chickpeas and lentils 50:50, PF), Barley milled grains (BMG)] on the human intestinal microbial ecosystem, using an in vitro three-stage continuous culture system simulating the human large intestine, which represents a relevant tool for monitoring the ecology and metabolic activities of colonic microbiota in relation to different external perturbations (Macfarlane et al., 1998; Macfarlane et al., 2011).

Main bacterial groups of the faecal microbiota were evaluated during the colonic model system study using the 16S rRNA-based FISH approach. Potential effects of supplementation of the four flours on the microbial physiology were studied using NMR-based metabolomics in combination with multivariate pattern recognition techniques (Bertini, et al., 2009; Bernini et al., 2011; Bertini et al., 2012). Finally, the ability of the metabolites of the cultural supernatants from the colonic model system, before and after the dietary supplementation, to modulate the growth of human intestinal epithelial cells was assessed using a HT29 cell-growth curve assay.

Substrates and simulated *in vitro* human digestion

Four different flours (WGR, NW, PF, BMG), whose nutritional profile was characterised by official reference methods (ashes: UNI ISO 2171; proteins: UNI 10274 831/12/93 and ISO 1871 (15/12/75); total dietary fibre: AOAC 985,29; RS: AOAC 2002.02; β-glucans: AOAC 995.16 2005; FOS: AOAC 997.08 and GOS: AOAC 2001.02), were selected considering their different composition and potential functional properties (Table 5). Prior to being added into the colon model system, the flours were digested *in vitro* under appropriate conditions according to the procedures described by Mills et al. (2008). Gastric and small intestinal environments were resembled using appropriate enzymatic concentration of HCl and pepsin, as well as bile salts and pancreatic enzymes, respectively. Dialyses with membrane of 100-200 Daltons cut off (Spectra/por 100-200 Da MWCO dialysis membrane, Spectrum Laboratories Inc.) were used to remove the monosaccharide from the pre-digested flours.

Flour	Ashes	Proteins	Sugars	TDF	RS	β-glucans	FOS	GOS
Wholegrain rye	1.55	8.91	1.91	18.04	< 0.20	1.89	4.50	1.53
Pulses	3.02	22.95	1.78	15.44	2.00	< 0.20	0.70	6.50
Nutriwheat	7.30	22.92	2.48	26.77	< 0.20	1.22	2.3	1.0
Barley milled grains	1.64	11.61	4.08	20.93	0.21	8.14	1.2	0.61

Table 5. Composition and nutritional profile of the four selected flours used in this study. Data are expressed as percentage of the total nutritional composition. TDF: Total dietary fibres; RS: Resistant starch.

Three-stage continuous culture gut model system

The three-stage continuous culture model of the human colon comprised of 3 glass fermenters of increasing working volume, simulating the proximal (V1, 280 mL), transverse (V2, 300 mL) and distal colon (V3, 320 mL). The 3 fermenters connected in series were kept at 37 °C, pH was maintained at 5.5 (V1), 6.2 (V2) and 6.8 (V3) and anaerobic conditions were introduced by continuously sparging with O_2-free N_2. V1 was fed by means of a peristaltic pump with CMGM (Macfarlane et al., 1998). Human faecal samples were collected on site, kept in an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2) and used within a maximum of 15 min after collection. This experiment was carried out in duplicate using faecal samples from two different healthy volunteers (1 male aged 31 and, 1 female; aged 31-38 years old). None of the volunteers had received antibiotics or probiotics for at least 3 months before sampling. A 1:5 (w/w) faecal dilution in anaerobic PBS [0.1 M PBS (pH 7.4)] was prepared and the samples homogenized in a stomacher (Seward, Worthing) for 2 min. Each stage of the colonic model was inoculated with 100 mL faecal slurry. Total system transit time was set at 48 h,

according to mean retention time of healthy individuals. Following inoculation, the colonic model was run as a batch culture for a 24 h period in order to stabilise bacterial populations prior to the initiation of medium flow. After 24 h (T0) the medium flow was initiated and the system ran for 8 full volume turnovers to allow for steady state to be achieved (SS1). Taking into account the operating volume (900 mL) and the retention time (48 h) of the colonic model system, dialysis retentate of the tested flours were added daily into V1 at 1% (w/v). The tested flours were added to the system as described for a further 8 volume turnovers upon which steady state 2 (SS2) was achieved. Each steady state was confirmed through sampling on three consecutive days for SCFAs and FISH analyses.

Sample collection and preparation

Samples for FISH were immediately fixed in 4% paraformaldehyde as previously described (Martín-Peláez et al., 2008).

Samples for HT29 cell growth curve analysis were centrifuged at 12,000 x *g* for 15 min and supernatants were sterile filtered and frozen immediately.

Samples for metabolomics analysis were homogenised at high speed in a mechanical homogeniser (MiniLab 8.30H, Rennie) for 30-45 s, further subjected to two cycles of freezing and thawing to breakdown cell membranes and release cytoplasmic metabolites, and finally centrifuged at 10,000 x *g* for 15 min at 4 °C. Supernatants were collected and stored at -80 °C until measurement.

In vitro enumeration of bacterial population by FISH

Numbers of the main intestinal bacterial groups, as well as total bacterial populations, was evaluated in samples from the colonic model system by FISH analysis, as previously

described by Martín-Peláez and colleagues. The probes used are reported in Annex 1 and were commercially synthesised and 5'-labelled with the fluorescent Cy3 dye (Sigma).

Modulation of HT29 cell growth by the tested flours

The influence of the colonic model supernatants, recovered before and after the supplementation of the four tested flours, on the growth and survival of the human colon carcinoma cell line HT29 was determined using the growth curve assay, as previously described by Maccaferri et al. (2010). Results are expressed as EC_{50}, which represents the effective concentration of colonic model supernatants resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions compared to the growth of untreated cells.

NMR profiling

Frozen samples were thawed at room temperature and centrifuged at 16,000 x *g* for 5 min. A total of 300 µl of a sodium phosphate buffer (70 mM Na_2HPO_4; 20% (v/v) ^2H_2O; 6.15 mM NaN_3; 6.64 mM TMSP (pH 7.4) was immediately added to 300 µl of each sample, and the mixture homogenised by vortexing for 30 s. NaN_3 was added to ensure that metabolites are not generated or consumed via the action of bacteria or bacterial enzymes during the time of NMR sample preparation and NMR spectra acquisition. A total of 450 µl of this mixture was transferred into a 4.25 mm NMR tube for analysis.

NMR spectra for all samples were acquired using a Bruker spectrometer (Bruker Biospin) operating at 14.1 T (600.13 MHz proton Larmor frequency) equipped with a CPTPI cryoprobe, an automatic tuning-matching unit and an automatic sample changer. A BTO 2000 thermocouple served for temperature stabilisation at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probe-

head, for temperature equilibration (27 °C). For each sample, a mono-dimensional (1D) NMR spectrum was acquired with water peak suppression using a standard pulse sequence (Bruker terminology: noesygppr1d.comp) (Le Gall et al., 2011), 64 scans, 96 k data points, a spectral width of 18028 Hz, and a relaxation delay of 4 s. Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were corrected for phase and baseline distortions and calibrated (proton signal of TMSP at 0.00 ppm) using TopSpin (Version 2.1; Bruker BioSpin). The regions between 5.0 and 4.5 ppm, which contain the residual water signal, were removed from the subsequent analysis. Each 1D spectrum in the range between 0.2 and 10.0 ppm was segmented into 0.02-ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX software (Version 3.8.4; Bruker BioSpin) giving a total of 466 variables. The total spectral area was calculated on the remaining bins and normalisation on the total area was carried out on the data prior to pattern recognition. All metabolites of interest were then checked and their NMR signals were assigned on template 1D NMR profiles by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (Version 2.0.0; Bruker BioSpin) reference databases and published literature (Le Gall et al., 2011; Wu et al., 2010; Bezabeh et al., 2009; Hong et al., 2011; Martin et al., 2010).

Statistical analysis

Bacterial counts and HT29 growth modulation data were analysed by one-way ANOVA, using Tukey's post-test analysis when the overall *P* value of the experiment was below the value of significance ($P < 0.05$). An additional paired t-test was applied in order to assess the significance of results of single pairs of data. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Metabolomic fingerprint-based data analysis

was carried out using R software with scripts developed in-house. Data were normalised to the total area and mean-centred before analysis (separately for each vessel). PCA was conducted in order to observe intrinsic clusters and a general overview of the variance of the NMR profiles. Furthermore, NMR spectra were subjected to O-PLS for highlighting the effects of the four different flours. O-PLS is an extension of the Partial Least Square regression method (Wold et al., 1984), featuring an integrated Orthogonal Signal Correction filter (Wold et al., 1998). The relative concentrations of the metabolites were calculated by integrating the signals in the spectra normalized to the total area. To evaluate the effects of different flours in the three vessels, the relative concentrations of the metabolites were mean centred (separately for each vessel). Statistical significance was assessed using univariate nonparametric Wilcoxon rank-sum test between SS1 and SS2 samples of each treatment. When $P < 0.05$, differences among samples were considered statistically significant.

Impact of the flours on the human colonic microbiota

Annex 4 describes the impact of the four flours on the composition of the main bacterial groups constituting the core colonic microbiota.

A number of significant modifications along the study have been shown for all the tested flours. In particular, WGR mediated a significant increase in concentration of *Bifidobacterium* genus, LAB and *Desulfovibrionales* spp. in all the stages of the colonic model system, whereas *Roseburia/E. rectale* group significantly decreased in V1, simulating the proximal colon, after the administration of this flour. Similarly, NW induced a significant increase in LAB in the first stage of the colonic model system, bifidobacteria in the second stage, simulating the transverse colon, and *Desulfovibrionales* spp. in the third stage, simulating the distal colon. A decrease in concentration of *Ruminococcus* spp. was further found in the whole colonic model system.

While modulating an overall increase of *Bacteroides/Prevotella* and a decrease of *Roseburia/E. rectale* group in the entire colonic model system, the supplementation of PF provoked several modifications in each region of the fermentative system: in V1, a decrease of *E. rectale/Clostridium* cluster XIVa group and *Atopobium* cluster, as well as a decrease of *Clostridium* cluster IX in V2, an increase of *Ruminococcus* spp. in V3 and a decrease of *F. prausnitzii* in V2 and V3 were demonstrated.

Finally, BMG provoked a decrease in *E. rectale/Clostridium* cluster XIVa group in the third stage of the colonic model system and *Roseburia/E. rectale* groups in the second and third stage. An increase of *Desulfovibrionales* spp. was concomitantly shown in the V1 and V2 when BMG was used as dietary flour.

HT29 growth modulation by the colonic model system supernatants

EC_{50} was used to compare the effect of colonic model supernatants, before and after the administration of the four different flours, on HT29 cell growth (Fig. 22). No significant changes between EC_{50}(SS1) and EC_{50}(SS2) were found in the all the stages composing the colonic model system, at SS1 and SS2, for any of the four tested dietary flours.

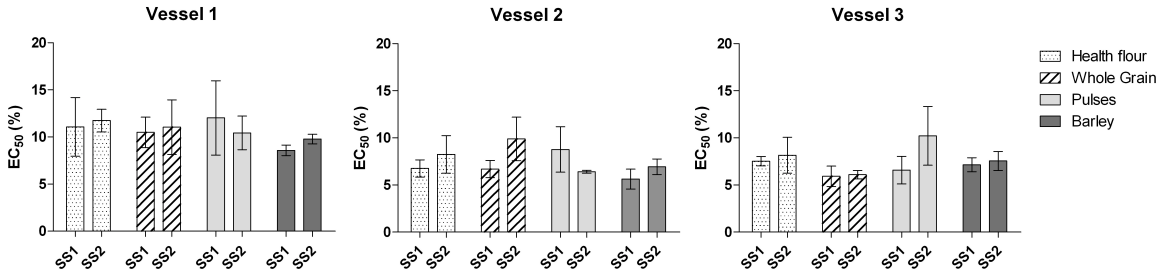


Figure 22. Effect of supernatants recovered from Vessel 1, Vessel 2, and Vessel 3 of the colonic model system, on HT29 growth before (SS1) and after (SS2) administration of the four dietary flours. Growth inhibition was assessed by co-incubating HT29 cells with increasing concentration (0%, 1%, 2.5%, 5%, 10%) of fermentation supernatants followed by DAPI staining. Results are expressed as means of relative HT29 cell growths (%) ± SEM of 2 colonic models with two independent sampling times each. For each colonic model and time point measurements were performed in triplicate. Figure shows the comparison of EC50 values in SS1 and SS2 for each vessel, for each of the four flours.

NMR analysis

The comparison of the NMR spectra (Figure 23) of samples from the colonic model at SS1 and SS2 samples (Table 6) demonstrated that all the tested dietary flours caused different modifications in metabolite concentrations. In particular, BMG and PF induced several changes in the metabolic profile. After the supplementation of BMG and PF, colon model metabolome was characterised by lower level of trimethylamine and higher level of acetate. In addition, PF provoked a decrease in levels of butyrate and isovalerate, whilst higher levels of propionate and tyrosine were found. Similarly to PF, NW induced lower level of butyrate and higher level of propionate. Interestingly, WGR induced a decrease in methanol not observed with any other flour.

PCA was performed to obtain a simplified view of the variation in the data and to understand the global metabolic changes in the three stages of the colon model. Two PC were calculated, PC1 and PC2, which explain 60.7% and 19.4% of the total variance, respectively. Scores along PC1 and PC2 showed a strong separation among the V1 samples and the V2 and V3 samples, as reported in Figure 24A. The outlier, found in the PCA plot, was due to the presence of a high concentration of lactate. O-PLS analysis was also performed on NMR profiles using as supervisory variable the different dietary interventions undertaken in the study (Figure 24B). This analysis demonstrated that the effect of PF and BMG supplementation on the on overall colonic metabolome was higher than that resulting from administration of NW and WGR.

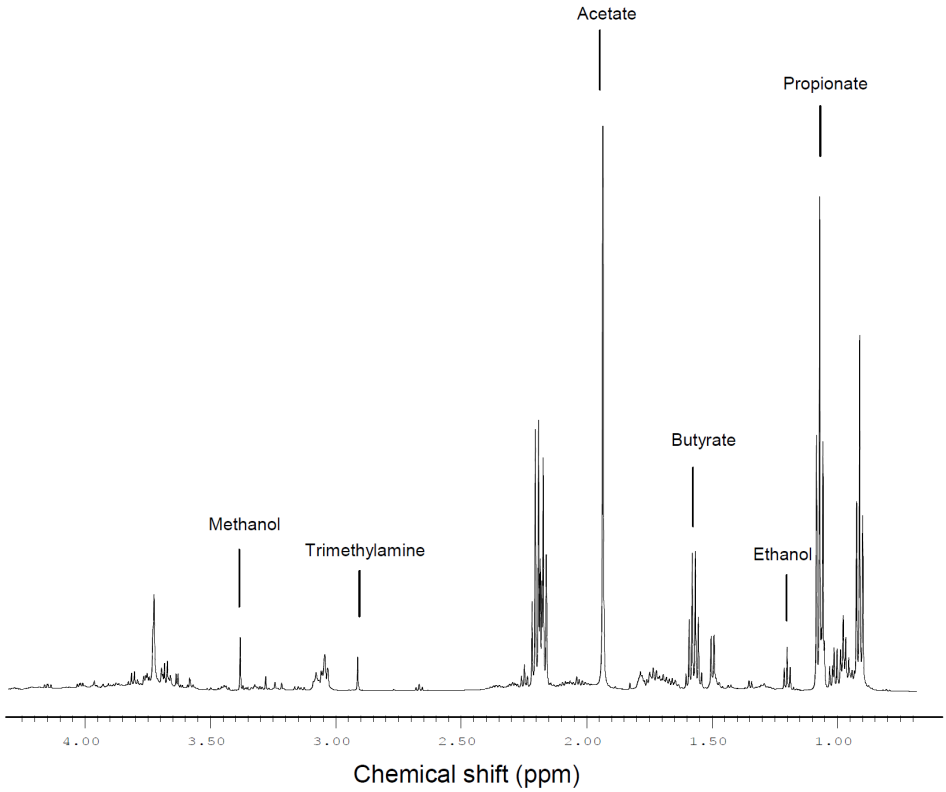
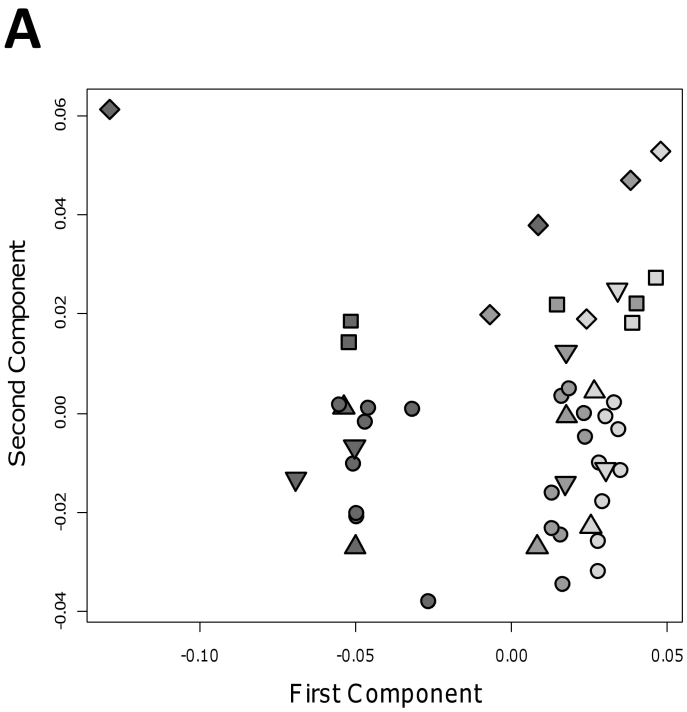


Figure 23. Spectral region 1.50–4.50 ppm of high resolution proton NMR 600 MHz spectra acquired with a 1D standard experiment from colonic model supernatants.

Compound	Pulses (PF)	Barley milled grains (BMG)	Nutriwheat (NW)	Wholegrain rye (WG)
Acetate	↑ **4.33 · 10-3**	↑ **2.16 · 10-3**	↓ 8.18 · 10-1	↑ 5.89 · 10-1
Alanine	↑ 6.49 · 10-2	↓ 6.99 · 10-1	↓ 6.99 · 10-1	↑ 5.89 · 10-1
Butyrate	↓ **2.16 · 10-3**	↓ 2.40 · 10-1	↓ **8.66 · 10-3**	↑ 6.99 · 10-1
Ethanol	↑ 6.99 · 10-1	↑ 3.10 · 10-1	↓ 3.10 · 10-1	↓ 5.89 · 10-1
Formate	↓ 6.99 · 10-1	↓ 8.18 · 10-1	↓ 9.37 · 10-1	↑ 1.32 · 10-1
Fumarate	↑ 6.99 · 10-1	↑ 3.94 · 10-1	↑ 1.00 · 10-0	↑ 2.40 · 10-1
Isoleucine	↑ 3.10 · 10-1	↓ 1.32 · 10-1	↑ 3.10 · 10-1	↑ 9.37 · 10-1
Isovalerate	↓ **2.60 · 10-2**	↑ 9.37 · 10-1	↓ 9.37 · 10-1	↑ 5.89 · 10-1
Lactate	↑ 6.99 · 10-1	↑ 1.00 · 10-0	↓ 3.94 · 10-1	↑ 2.40 · 10-1
Leucine	↑ 3.94 · 10-1	↓ 6.99 · 10-1	↑ 2.40 · 10-1	↑ 8.18 · 10-1
Lysine	↑ 1.32 · 10-1	↑ 9.37 · 10-1	↑ **2.16 · 10-3**	↓ 6.99 · 10-1
Methanol	↑ 1.80 · 10-1	↓ 3.94 · 10-1	↓ 5.89 · 10-1	↓ **2.16 · 10-3**
Phenylalanine	↑ 6.49 · 10-2	↑ 3.10 · 10-1	↑ 1.32 · 10-1	↑ 1.00 · 10-0
Propionate	↑ **2.60 · 10-2**	↓ 1.00 · 10-0	↑ **2.16 · 10-3**	↓ 3.10 · 10-1
Trimethylamine	↓ **4.11 · 10-2**	↓ **8.66 · 10-3**	↓ 1.80 · 10-1	↓ 1.80 · 10-1
Tyrosine	↑ **4.11 · 10-2**	↑ 3.10 · 10-1	↑ 1.32 · 10-1	↓ 3.94 · 10-1
Uracil	↑ 9.37 · 10-1	↑ 5.89 · 10-1	↓ 1.52 · 10-2	↓ 8.18 · 10-1
Valine	↑ 6.99 · 10-1	↓ 6.49 · 10-2	↑ 9.37 · 10-1	↓ 8.18 · 10-1

Table 6. *P* Values of metabolites resulting from the comparison between control and different dietary interventions. Significant differences between SS1 and SS2 are highlighted in bold and underlined.



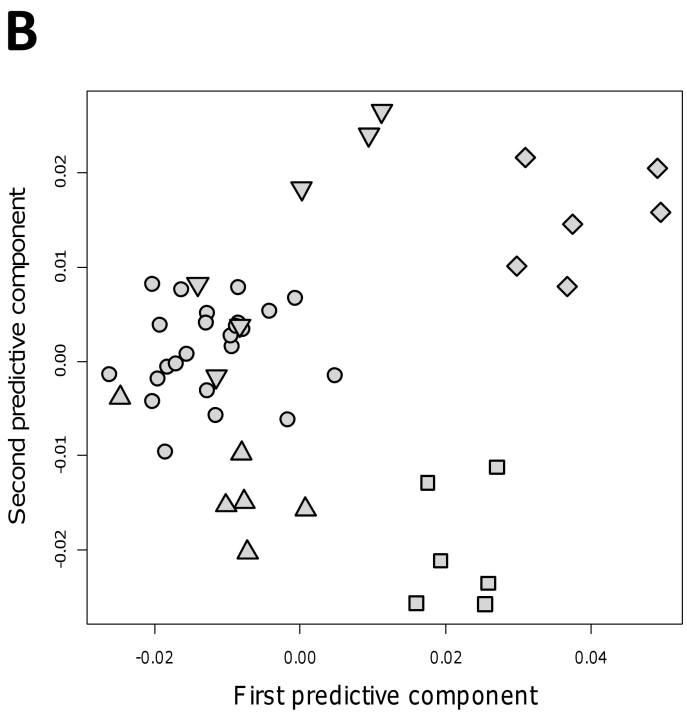


Figure 24. Multivariate analysis of 1H-NMR spectra of faecal samples. (A) PCA scores plot coloured according to the vessel: dark grey Vessel 1, grey Vessel 2, and light grey Vessel 3. (B) O-PLS scores plot discriminating the effects of different probiotics. Circle SS1, square PF, rhombus BMG, triangle NW, inverted triangle WGR rye.

CHAPTER 5 - *IN VITRO* FERMENTATION OF NOVEL DIETARY FIBRES

In recent years, a new health paradigm has evolved, placing more emphasis on the beneficial aspects of diet. Although the primary role of diet is to provide nutrients to fulfil metabolic requirements, the use of foods to improve health and wellbeing is being increasingly accepted (Figueroa-Gonzalez et al., 2011).

Dietary fibres and prebiotics can be included in a wide range of foods, such as bakery, dairy and beverage products. In particular, prebiotics are oligosaccharides or more complex saccharides that are selectively metabolised by some commensal groups, including those considered to be beneficial for the host, thus impacting on the gut microbiota composition and functional activity (Sherman et al., 2009; Laparra and Sanz, 2009).

Cereal grains can contain naturally occurring oligosaccharides such as galactosyl derivatives of sucrose stachyose and raffinose and fructosyl derivatives of sucrose. Furthermore, cereal grains and pulses contain dietary fibre, which encompasses a heterogeneous range of complex polysaccharides that are not substantially digested in the small intestine and pass through to the colon. Therefore, dietary fibre constitutes a potential and not yet fully explored source of prebiotics from cereals and legumes (Charalampopoulos et al., 2002).

In the perspective of designing foods from natural sources and more effective dietary strategies for human health promotion, here we studied the putative prebiotic potential of four flours, which are naturally rich in fibre, by assessing their impact on the human intestinal microbial ecosystem.

The four tested flours belong to two different categories, cereal grain flours, namely wholegrain rye, wholegrain wheat and barley milled grains, and pulses flour, which are not yet evaluated for their prebiotic potential. In particular, the two wholegrain flours, one deriving from rye and one from wheat, contain naturally high concentrations of GOS and FOS, whilst the pulses flour, composed by a mix of lentils and chickpeas 50:50, is particularly rich in GOS and RS. Finally, the barley milled grains flour is a rich source for β-glucans.

β-glucans are components of dietary cereals that are becoming increasingly recognized as functional ingredients in food and drink products (Naumann et al., 2006). Mixed-linkage β-glucans, present at high levels in the BMG flour, have been demonstrated to evoke a range of metabolic and physiological responses, as lowering cholesterol levels and insulin responses (Naumann et al., 2006; Kerckhoffs et al., 2003). In the present study, an increase in acetate, following BMG administration in the colon model system, has been demonstrated. Our results support positive modulation of SCFA by barley, as previously reported by Hughes et al. (2008), who evaluated the *in vitro* fermentation of barley-derived β-glucans by the human faecal microbiota, demonstrating a significant increase in propionate. Notably, we demonstrated a decrease in the concentration of trimethylamine, a precursor of the trimethylamine N-oxide. This harmful compound, produced by commensal inhabitants of the intestinal microbiota, has been demonstrated to play a role in the atherogenesis (Loscalzo, 2011).

Pulses contain a number of bioactive substances including enzyme inhibitors, lectins, phytates, oligosaccharides and phenolic compounds. These polyphenolic compounds consist mainly of tannins, phenolic acids and flavonoids (Campos-Vega et al., 2009). Phenolics are metabolized by the gut microbiota, affecting intestinal health (Selma et al., 2009). In addition, chickpeas, which contain significant levels of oligosaccharides, non-starch polysaccharides, RS and resistant protein can exert a not yet fully explored prebiotic potential (Sanchez-Mata

et al., 1998). Here, we demonstrated that PF induces an extensive modulation of the colonic metabolome, provoking an increase in concentration of acetate and propionate, as well as a decrease of butyrate and branched-chain fatty acid iso-valerate. This metabolic shift can be explained by the overall increase of *Bacteroides/Prevotella* species, known to be primary propionate and acetate producers, which are representing a considerable share of the intestinal microbiota resulting from the supplementation with PF. Conversely, the decrease of major butyrate-producers as *Clostridium* cluster IV and XIVa can be related to the decrease of butyrate (Charalampopoulos and Rastall, 2009). Notably, propionate is a major microbial fermentation metabolite in the human gut with well-known health effects not only at colon level, but also in a broader human body context. In fact, propionate is thought to lower lipogenesis and serum cholesterol level, as well as to play a role in weight control by stimulating satiety (Hosseini et al., 2011).

Wholegrain has been extensively studied for its beneficial effects and several epidemiological studies have shown its protective activity against cancer, diabetes, obesity and CVD (Katcher et al., 2008; Slavin, 2004). However, little is known about peculiar modulation of the colonic microbiota related to wholegrain rye and wheat. In the present study, we have demonstrated that WGR impacts on the colonic microbiota, eliciting an increase in concentration of bifidobacteria, lactobacilli and *Desulfovibrionales*, whilst a decrease in *Roseburia* genus was observed. Similarly, NW caused significant yet less pronounced increase of bifidobacteria, LAB and *Desulfovibrionales* in comparison with WGR, whereas a decrease of ruminococci was further demonstrated. These data are in accordance with those of Costabile et al. (2008), who for the first time broadly investigated the impact of wholegrain on the human colonic microbiota. In particular, these authors found significant increases in lactobacilli/enterococci after the ingestion of either wheat bran or wholegrain but in *Bifidobacterium* spp. only after wholegrain consumption.

The supplementation of wholegrain-based flours (WGR and NW) induced modulation of the colonic metabolome. Indeed, we demonstrated a decrease in the concentration of methanol, a compound that has been previously demonstrated to increase in a number of pathological conditions, especially in subjects affected by *Campylobacter jejuni* and ulcerative colitis (Garner et al., 2007). A decrease in methanol concentration related to a modulation of the colonic microbiota towards a more bifidogenic structure has been reported in literature. In fact, a decrease in this alcohol has been associated with positive and bifidogenic modulation of the colonic microbiota of patients affected by Crohn's diseases after treatment with the antibiotic rifaximin (Maccaferri et al., 2010). Conversely, no significant changes in concentrations of the SCFAs acetic, propionic, butyric or caproic acids were observed in response to WGR administration.

NMR analysis of the colon model metabolome following the NW administration depicted a different scenario. NW is mediating a decrease of butyrate and an increase of propionate and the essential amino acid lysine. Notably, the decrease in concentration of butyrate could be explained by a depletion of *Ruminococcus* spp. after the supplementation of the wholegrain wheat flour, since ruminococci are well-known to produce butyrate (De Vuyst et al., 2011).

Growth inhibition of colon carcinoma cells, differentiation and apoptosis are thought to be mechanisms by which dietary fibre exerts a chemopreventive effect in the colon (Lupton, 2004). However, the administration of all the four flours from natural sources did not influence the growth-modulatory potential of the supernatants recovered from the three stages of the colonic model system. Since it has been demonstrated that the concentration of butyrate in fermentation supernatants from colonic model systems was directly associated with their growth inhibitory potential (Klinder et al., 2004), our observations in the HT29 growth assay are in accordance with the NMR results showing that the concentration of butyrate in the supernatants did not change or even decreased after administration of the flours. In

conclusion, wholegrain rye and wheat, pulses and barley milled grain flours showed peculiar and positive modulations of the intestinal microbiota composition and its small molecule metabolome. Therefore, our results could support the utilisation of these ingredients in the development of a variety of potentially prebiotic food products aimed at improving gastrointestinal health.

CONCLUDING REMARKS

CHAPTER 6 - CONCLUDING REMARKS

The utilisation of probiotics and prebiotics to promote human health is increasing, and a solid scientific substantiation of the health claims associated to this food ingredients has to be generated in order to develop new functional foods is required.

In this study, we extensively evaluated the potential probiotic yeast *K. marxianus* B0399. We presented a combined *in vitro* and *in vivo* approach for the evaluation of the beneficial activities of the novel probiotic strain *K. marxianus* B0399, which included a preliminary broad investigation of the intrinsic characteristics of the yeast, followed by a human pilot study for evaluating its impact on the human intestinal microbiota.

We demonstrated that *K. marxianus* B0399 possesses a number of beneficial and strain-specific properties, which are desirable in a microorganism to be considered for application as a probiotics, i.e. strong adhesion to intestinal epithelial cells, immune modulation and impact on the composition of the colonic microbiota. Furthermore, we demonstrated that unbalances in the composition of the intestinal microbiota of subjects suffering from IBS are not counterbalanced by the administration of a probiotic yoghurt containing *K. marxianus* B0399 and *B. animalis* subsp. *lactis* Bb12. However, the administration of the diary probiotic formulation investigated in the present study has been demonstrated to be beneficial in the management of IBS symptoms. Therefore, these findings enforce the necessity of characterising the mechanism of action of clinically relevant probiotic strains not only towards the composition of the gut microbiota, but also taking into account its functionality.

Besides probiotics, prebiotics are gaining a relevant role in the dietary promotion of human health, due to their ability to influence the symbiotic intestinal microbiota. In the perspective

of designing foods from natural sources and more effective dietary strategies for human health promotion, in the present study we further investigated studied the putative prebiotic potential of three cereal grain flours and one pulses flour.

Combining a molecular and cellular approach with cutting-edge metabolomics methods, we demonstrated that wholegrain rye and wheat, pulses and barley milled grain flours showed different and peculiar positive modulations of the intestinal microbiota composition and its small molecule metabolome. Therefore, our results could support the utilisation of these ingredients in the development of a variety of potentially prebiotic food products to be tested *in vivo* for substantiating claims on the gastrointestinal health promotion.

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ACKNOWLEDGMENTS

CHAPTER 8 - ACKNOWLEDGMENTS

I have to admit it is not an easy task to acknowledge and mention every person who has been meaningful in the last three year. Not because my memory is getting rarer - OK, I'm ageing, but not that much - but just because I was so lucky to go through a huge number of life experiences.

I cannot start from anyone else than my lab mates, who shared with me much more than science: good feelings, mutual support, long discussions on the future expectations, political analyses about the uncertain present, and - more concretely - also common room and desks, not a minor issue, indeed. And obviously a word for Patrizia, because the 24/7 lifestyle experience will be an "odd memento" also in 2020.

However, every time I'm tracing back my experiences in the last three years, I leave a piece of my essence in that grey and rainy place up in the North. I had the chance to meet many friends and to keep in touch with them again - Reading it's a kind of hideaway where I'm living quietly and far away from the daily routine, every time I'm catching a flight to go there. It would be great to share again these good sensations in the future. For sure, it will be great to have you around during my dissertation, physically or just with your soul and your greetings.

Finally, I'd like to spend a couple of words for all that people who made possible for me to be so multitasking, filling some gaps during my breaks and my many personal activities. Being part of a good team it's not just working hard for yourself, but to give support to all the people who are next to you.

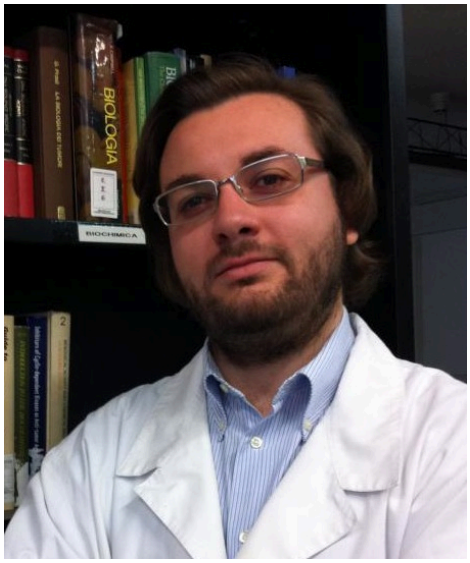
And what's next? The time to find a definite future for me is not yet arrived. I hope, it will be bright. Anyway, and anywhere. Also because of you.

Cheers,

Simone

BIOGRAPHY

CHAPTER 9 - BIOGRAPHY



Simone Maccaferri graduated as a Pharmaceutical Biotechnologist at the University of Bologna, in 2008, after completing a visiting period of 8 months at the Department of Food and Nutritional Sciences, University of Reading (United Kingdom). His research activity is mainly focused on microbial ecology of the human gastrointestinal tract. After his graduation, he worked as research assistant for Alfawasserman Spa, Bologna, Italy. In 2009, Simone was admitted to attend a European PhD Programme in "Microbiology and Industrial Biotechnology" at the University of Bologna, leading the project: "Characterization of novel probiotics and prebiotics". His research activity was resulting from two collaborative project, which involved international partners from academia and industry. In 2010, Simone has been visiting PhD Student for 6 months at the Department of Food and Nutritional Sciences, University of Reading (United Kingdom).

Simone is currently affiliated member of the International Scientific Association for Probiotics and Prebiotics (ISAPP) and is full member of the European Network for Gastrointestinal Health Research (ENGIHR).

Since March 2009, Simone is the President of the Association of the Italian Biotechnologists (ANBI) and he is chairing the Scientific Board of the magazine "Prometeus – ANBI Magazine". In 2005-2007 he was member of the FP6 European Project BIOPOP consortium, aimed to develop new communication format and to promote participation of citizens in discussion about life sciences. Furthermore, he is member of the Advisory Board of Degree/ Post-graduate Master in Biotechnology of 7 Italian Universities.

List of Selected Publications

CHAPTER 10 - LIST OF SELECTED PUBLICATIONS

1. Maccaferri S, Klinder A, Cacciatore S, Chitarrari R, Honda H, Luchinat C, Bertini I, Carnevali P, Gibson GR, Brigidi P, Costabile A: **In vitro fermentation of potential prebiotic flours from natural sources: impact on the human colonic microbiota and metabolome**. Submitted to *Molecular Nutrition and Food Research.*

2. Maccaferri S, Candela M, Turroni S, Centanni M, Severgnini M, Consolandi C, Cavina P, Brigidi P: **IBS-associated phylogenetic unbalances of the intestinal microbiota are not reverted by probiotic supplementation**. Submitted to *Gut Microbes.*

3. Candela M, Biagi E, Maccaferri S, Turroni S, Brigidi P: **Intestinal microbiota is a plastic factor responding to environmental changes.** Submitted to *Trends in Microbiology.*

4. Vitali B, Ndagijimana M, Maccaferri S, Biagi E, Guerzoni ME, Brigidi P: **An *in vitro* evaluation of the effects of probiotics and prebiotics on the metabolic profile of healthy-like human microbiota.** Submitted to *Anaerobe.*

5. Maccaferri S, Klinder A, Brigidi P, Cavina P, Costabile A: **Potential Probiotic Kluyveromyces marxianus B0399 Modulates the Immune Response in Caco-2 Cells and Peripheral Blood Mononuclear Cells and Impacts the Human Gut Microbiota in an In Vitro Colonic Model System.** *Applied and Environmental Microbiology* 2012, **78**:956-64.

6. Vitali B, Minervini G, Rizzello CG, Spisni E, Maccaferri S, Brigidi P, Gobbetti M, Di Cagno R: **Novel probiotic candidates for humans isolated from raw fruits and vegetables.** *Food Microbiology* 2012 in press, http://dx.doi.org/10.1016/j.fm.2011.12.027

7. Maccaferri S, Biagi E, Brigidi P: **Metagenomics: key to human gut microbiota.** *Digestive diseases* 2011, **29**:525-30.

8. Maccaferri S, Vitali B, Klinder A, Kolida S, Brigidi P, Costabile A: **Rifaximin modulates the colonic microbiota of patients with Crohn's disease: an in vitro approach using a continuous culture colonic model system - authors' response.** *The Journal of Antimicrobial Chemotherapy* 2011, **66**: 1194-1195.

9. Maccaferri S, Vitali B, Klinder A, Kolida S, Ndagijimana M, Laghi L, Calanni F, Brigidi P, Gibson GR, Costabile A: **Rifaximin modulates the colonic microbiota of patients with Crohn's disease: an in vitro approach using a continuous culture colonic model system.** *The Journal of Antimicrobial Chemotherapy* 2010, **65**:2556-65.

10. Candela M, Maccaferri S, Turroni S, Carnevali P, Brigidi P: **Functional intestinal microbiome, new frontiers in prebiotic design.** *International Journal of Food Microbiology* 2010, **140**:93-101.

ANNEXES

Annex 1. Oligonucleotide probes used in this study for FISH analysis.

Target genus or group	Probe	Sequence (5' to 3')
Most Bacteria	EUB338[a]	GCTGCCTCCCGTAGGAGT
Most Bacteria	EUB338II[a]	GCAGCCACCCGTAGGTGT
Most Bacteria	EUB338III[a]	GCTGCCACCCGTAGGTGT
Atopobium, Colinsella, Olsenella and Eggerthella spp.*; Cryptobacterium curtum; Mycoplasma equigenitalium* and *Mycoplasma elephantis*	Ato291	GGTCGGTCTCTCAACC
Most *Bacteroides* sensu stricto and *Prevotella* spp.; all *Parabacteroides*; *Barnesiella viscericola* and *Odoribacter splanchnicus*	Bac303	CCAATGTGGGGGACCTT
Most *Bifidobacterium* spp	Bif164	CATCCGGCATTACCACCC
Most *Deltaproteobacteria* and most *Gemmatimonadetes*	DELTA495a[b]	AGTTAGCCGGTGCTTCCT
Some *Deltaproteobacteria*	DELTA495b[b]	AGTTAGCCGGCGCTTCCT
Some *Deltaproteobacteria*	DELTA495c[b]	AATTAGCCGGTGCTTCCT
Most members of *Clostridium* cluster XIVa; *Syntrophococcus sucromutans, [Bacteroides] galacturonicus* and *[Bacteroides] xylanolyticus, Lachnospira pectinschiza* and *Clostridium saccharolyticum*	Erec482	GCTTCTTAGTCARGTACCG
Faecalibacterium prausnitzii and related sequences	Fprau655	CGCCTACCTCTGCACTAC
Most *Lactobacillus, Leuconostoc* and *Weissella* spp.*; Lactococcus lactis;* all *Vagococcus, Enterococcus, Melisococcus, Tetragenococcus, Catellicoccus, Pediococcus* and *Paralactobacillus* spp.	Lab158[#]	GTATTAGCAYCTGTTTCCA
Most members of *Clostridium* cluster I; all members of *Clostridium* cluster II; *Clostridium tyrobutyricum; Adhaeribacter aquaticus* and *Flexibacter canadensis (*family *Flexibacteriaceae); [Eubacterium] combesii (*family *Propionibacteriaceae)*	Chis150	TTATGCGGTATTAATCTYCCTTT
Clostridium cluster IX	Prop853	ATTGCGTTAACTCCGGCAC
Roseburia subcluster	Rrec584	TCAGACTTGCCG(C/T)ACCGC

Target genus or group	Probe	Sequence (5' to 3')
Most *Desulfovibrionales*	DSV567	TACGGATTTCACTCCT
Clostridium sporosphaeroides, Ruminococcus bromii, Clostridium leptum	Rbro730[c #]	TAAAGCCCAGCYAGGCCGC
Ruminococcu salbus, Ruminococcus flavefaciens	Rfla729[c #]	AAAGCCCAGTAAGCCGCC

[a, b, c] These probes were used in equimolar concentrations

[#] These probes were used following pre-treatment with Lysozyme (100U; 20 μL of 1 mg/mL solution of 50,000 U/ mg protein)

Annex 2. Sequence of the excised and re-amplified band referred to *K. marxianus*

TCAAACTGTTAAAAAATAACTGGGTTCCTCGCCaCACGGGATTCTCACCCTCTATGA

CGTCCTGTTCCAGGAACATAGACAAGGACGAGCTACAAAGTCGCCTTCTTCAAAT

TACAACTCGGACGTCGAAGACGCCAGATTTCAAATTTGAGCTTTTGCCGCTTCACT

CGCCGTTACTAAGGCAATCCCGGTTGGTTTCTTTTCCTCCGCTTATTGATATGGCCC

GCCGCCGGCTCGCGCGTCCCGGCGGTCGCGCGGCCCGCA

Annex 3. Data of relative abundance of main bacterial groups targeted by HTF-Microbi.Array in subjects suffering from IBS and healthy individuals. Data are expressed as percentage of each bacterial group on the total targeted microbiota

Index	Bacterial group/species	Index	Bacterial group/species
1	*B. longum*	18	*B. breve*
2	*Y. enterocolitica*	19	*B. adolescentis*
3	*Proteus* spp.	20	*B. bifidum*
4	*Campylobacter* spp.	21	*B. clausii*
5	*B. cereus*	22	*L. plantarum*
6	*B. subtilis*	23	*L. buchnerii*
7	*E. faecalis*	24	*Clostridium* cluster *I-II*
8	*E. faecium*	25	*Clostridium* cluster *XI*
9	*L. casei*	26	*Clostridium* cluster *IX*
10	*L. salivarius*	27	*R. bromii*
11	*Fusobacterium* spp.	28	*R. albus*
12	*Bacteroides/Prevotella*	29	*F. prausnitzii*
13	*C. perfringens*	30	*O. guillermondii*
14	*C. difficile*	31	*Cyanobacteria* spp.
15	*E. rectale*	32	*Lactobacillaceae*
16	*Veillonella* spp.	33	*Enterobacteriaceae*
17	*Clostridium* cluster *XIV*	34	*Bidifobacteriaceae*

Subjects suffering from IBS, enrolled in the study:

	1	2	3	4	5	6	7	8	9
201_t0	1.39	1.34	1.43	1.11	1.27	1.15	1.29	1.09	1.08
201_t1	0.67	0.85	1.01	0.96	0.92	0.66	0.77	0.71	0.62
27_t0	1.02	0.94	0.87	0.81	0.99	0.91	1.00	0.86	0.86

	1	2	3	4	5	6	7	8	9
27_t1	0.98	1.04	1.06	1.03	1.28	0.98	1.01	1.19	1.08
28_t0	1.14	1.08	1.08	1.17	1.36	1.37	1.02	0.91	0.94
28_t1	1.00	0.83	0.83	0.66	1.16	0.72	1.07	0.72	0.67
31_t0	0.66	0.81	1.93	0.63	0.93	1.21	1.87	0.60	0.68
31_t1	0.33	0.44	2.95	0.33	0.38	2.69	1.75	0.29	0.29
32_t0	0.83	1.31	0.87	0.63	1.37	0.75	0.68	0.69	0.76
32_t1	0.64	1.41	1.05	0.70	2.49	0.86	0.70	0.57	0.51
33_t0	1.25	1.33	0.97	0.88	1.30	0.83	0.96	1.18	0.90
33_t1	0.96	0.93	0.59	0.51	0.96	0.50	0.57	0.51	0.63
34_t0	0.58	0.77	0.97	0.63	0.96	0.67	0.73	0.75	0.65
34_t1	0.68	0.79	0.87	0.74	1.18	1.02	0.76	0.67	0.65
35_t0	0.77	0.93	1.02	0.96	0.96	0.74	0.73	0.84	0.81
35_t1	0.76	0.88	0.86	0.69	0.74	0.77	0.70	0.71	0.67
37_t0	0.50	0.57	0.56	0.38	1.23	0.66	0.48	0.58	0.38
37_t1	0.50	1.75	0.66	0.37	1.59	0.41	0.40	0.54	0.64
59_t0	0.60	0.47	2.53	0.24	0.61	2.09	0.96	1.11	0.24
59_t1	0.97	1.24	1.06	0.75	1.80	0.77	0.75	0.65	0.70
63_t0	1.06	0.59	2.05	0.26	0.60	1.29	1.02	1.61	1.99
63_t1	0.24	0.81	0.71	0.67	1.66	1.63	0.51	0.45	0.69
65_t0	0.58	0.56	0.49	0.39	0.99	0.44	0.68	0.63	0.54
65_t1	0.90	1.51	1.07	0.70	1.42	0.73	2.21	0.79	1.02
66_t0	0.26	0.21	2.02	0.60	0.17	1.45	1.48	1.10	0.17
66_t1	1.76	1.56	1.50	1.28	2.07	1.41	1.34	1.25	1.30
78_t0	0.67	0.64	0.78	0.56	0.65	0.71	0.63	0.71	0.60
78_t1	1.77	2.07	1.73	1.55	1.80	2.03	1.81	1.60	1.66
80_t0	0.24	0.32	1.76	0.25	0.24	1.42	1.33	0.27	0.17
80_t1	0.65	0.74	0.70	0.55	0.84	0.66	0.59	0.59	0.60
82_t0	1.17	1.56	1.18	1.13	1.27	1.17	1.42	1.24	1.22
82_t1	0.97	1.29	1.18	0.92	1.15	1.02	0.94	1.00	0.96
83_t0	0.95	1.35	1.02	0.37	1.33	1.29	0.74	1.09	0.25

	1	2	3	4	5	6	7	8	9
83_t1	1.18	0.98	1.80	1.00	1.23	0.58	0.40	1.69	0.23
84_t0	0.67	1.39	1.30	0.59	2.41	0.89	0.64	0.64	0.60
84_t1	1.02	1.67	1.59	0.76	2.14	1.06	0.90	0.96	0.90
89_t0	0.89	0.90	0.58	0.61	1.16	0.67	0.54	0.72	0.72
89_t1	2.00	1.99	1.87	1.27	2.80	1.46	1.84	1.17	1.13

	10	11	12	13	14	15	16	17	18
201_t0	3.57	1.59	11.24	1.83	1.07	3.60	4.55	10.58	1.10
201_t1	0.68	0.95	26.40	1.24	0.61	4.97	2.42	8.18	0.79
27_t0	0.93	1.27	11.55	0.92	0.79	6.63	5.94	9.94	0.85
27_t1	1.50	1.20	5.73	1.17	1.08	6.96	6.71	16.05	0.98
28_t0	1.01	1.25	10.87	1.30	1.25	8.09	3.21	9.18	1.16
28_t1	4.13	0.83	3.48	0.71	0.72	15.51	3.62	21.80	0.72
31_t0	0.86	1.79	8.95	0.90	0.60	3.97	9.44	11.95	0.79
31_t1	1.49	1.61	13.21	0.73	0.29	3.70	5.92	2.23	0.32
32_t0	1.50	0.93	3.41	0.82	0.60	9.19	4.91	29.78	0.67
32_t1	1.10	1.23	8.22	0.86	0.55	6.54	9.01	26.57	0.79
33_t0	1.97	1.30	5.08	1.02	0.83	6.69	1.70	23.17	1.03
33_t1	0.72	0.78	4.37	0.61	0.76	12.08	2.04	30.09	0.54
34_t0	3.10	0.80	14.86	1.01	0.68	10.06	4.34	14.17	0.86
34_t1	2.53	0.96	10.55	1.08	0.69	6.41	3.51	12.91	0.66
35_t0	1.07	0.98	4.18	1.22	0.75	4.80	3.44	23.53	0.82
35_t1	3.19	0.71	2.02	0.90	0.68	5.75	4.09	21.93	0.88
37_t0	0.39	0.85	17.57	0.62	0.35	6.79	5.79	26.82	0.37
37_t1	0.82	0.56	9.37	0.52	0.58	12.29	2.73	32.73	0.41
59_t0	0.41	3.68	12.58	0.52	0.18	6.42	11.54	7.68	0.25
59_t1	1.03	1.39	6.64	0.88	0.57	13.60	5.20	22.80	0.59
63_t0	2.14	2.39	6.37	1.62	0.60	12.73	6.15	11.50	0.64
63_t1	1.79	1.01	6.89	5.28	0.31	8.22	2.22	18.11	1.74
65_t0	1.39	0.68	14.29	0.56	0.40	6.13	2.45	21.46	0.42

	10	11	12	13	14	15	16	17	18
65_t1	1.27	0.91	3.53	1.54	0.91	4.52	2.37	15.05	1.01
66_t0	2.06	1.89	10.59	0.46	0.16	2.98	3.52	9.37	0.17
66_t1	1.39	1.81	9.67	1.72	1.17	2.74	4.26	14.12	1.48
78_t0	0.96	3.15	11.45	0.69	0.62	5.90	15.45	9.60	0.63
78_t1	1.80	1.66	5.16	1.85	1.71	6.75	2.08	15.74	2.25
80_t0	0.44	2.96	7.77	0.49	0.16	3.68	8.01	7.59	0.18
80_t1	1.82	0.75	10.63	0.75	0.53	2.71	2.38	15.10	0.64
82_t0	1.26	1.47	6.91	1.57	1.34	3.09	2.02	14.89	1.23
82_t1	0.99	1.11	1.72	1.40	1.32	3.92	4.06	18.63	1.05
83_t0	0.92	0.75	6.31	1.20	0.44	8.17	3.59	9.91	0.84
83_t1	7.72	1.58	11.71	0.97	0.40	3.14	3.12	20.22	0.32
84_t0	5.06	1.56	7.49	0.89	0.55	10.80	10.13	20.41	0.68
84_t1	4.40	1.65	8.68	1.18	0.94	8.90	7.45	17.55	1.05
89_t0	0.62	0.61	1.55	0.70	0.69	3.99	1.32	35.18	0.64
89_t1	1.36	1.50	4.53	1.64	1.37	3.60	10.86	9.37	1.64

	19	20	21	22	23	24	25	26	27
201_t0	3.57	1.59	11.24	1.83	1.07	3.60	4.55	10.58	1.10
201_t1	0.68	0.95	26.40	1.24	0.61	4.97	2.42	8.18	0.79
27_t0	0.93	1.27	11.55	0.92	0.79	6.63	5.94	9.94	0.85
27_t1	1.50	1.20	5.73	1.17	1.08	6.96	6.71	16.05	0.98
28_t0	1.01	1.25	10.87	1.30	1.25	8.09	3.21	9.18	1.16
28_t1	4.13	0.83	3.48	0.71	0.72	15.51	3.62	21.80	0.72
31_t0	0.86	1.79	8.95	0.90	0.60	3.97	9.44	11.95	0.79
31_t1	1.49	1.61	13.21	0.73	0.29	3.70	5.92	2.23	0.32
32_t0	1.50	0.93	3.41	0.82	0.60	9.19	4.91	29.78	0.67
32_t1	1.10	1.23	8.22	0.86	0.55	6.54	9.01	26.57	0.79
33_t0	1.97	1.30	5.08	1.02	0.83	6.69	1.70	23.17	1.03
33_t1	0.72	0.78	4.37	0.61	0.76	12.08	2.04	30.09	0.54
34_t0	3.10	0.80	14.86	1.01	0.68	10.06	4.34	14.17	0.86

	19	20	21	22	23	24	25	26	27
34_t1	2.53	0.96	10.55	1.08	0.69	6.41	3.51	12.91	0.66
35_t0	1.07	0.98	4.18	1.22	0.75	4.80	3.44	23.53	0.82
35_t1	3.19	0.71	2.02	0.90	0.68	5.75	4.09	21.93	0.88
37_t0	0.39	0.85	17.57	0.62	0.35	6.79	5.79	26.82	0.37
37_t1	0.82	0.56	9.37	0.52	0.58	12.29	2.73	32.73	0.41
59_t0	0.41	3.68	12.58	0.52	0.18	6.42	11.54	7.68	0.25
59_t1	1.03	1.39	6.64	0.88	0.57	13.60	5.20	22.80	0.59
63_t0	2.14	2.39	6.37	1.62	0.60	12.73	6.15	11.50	0.64
63_t1	1.79	1.01	6.89	5.28	0.31	8.22	2.22	18.11	1.74
65_t0	1.39	0.68	14.29	0.56	0.40	6.13	2.45	21.46	0.42
65_t1	1.27	0.91	3.53	1.54	0.91	4.52	2.37	15.05	1.01
66_t0	2.06	1.89	10.59	0.46	0.16	2.98	3.52	9.37	0.17
66_t1	1.39	1.81	9.67	1.72	1.17	2.74	4.26	14.12	1.48
78_t0	0.96	3.15	11.45	0.69	0.62	5.90	15.45	9.60	0.63
78_t1	1.80	1.66	5.16	1.85	1.71	6.75	2.08	15.74	2.25
80_t0	0.44	2.96	7.77	0.49	0.16	3.68	8.01	7.59	0.18
80_t1	1.82	0.75	10.63	0.75	0.53	2.71	2.38	15.10	0.64
82_t0	1.26	1.47	6.91	1.57	1.34	3.09	2.02	14.89	1.23
82_t1	0.99	1.11	1.72	1.40	1.32	3.92	4.06	18.63	1.05
83_t0	0.92	0.75	6.31	1.20	0.44	8.17	3.59	9.91	0.84
83_t1	7.72	1.58	11.71	0.97	0.40	3.14	3.12	20.22	0.32
84_t0	5.06	1.56	7.49	0.89	0.55	10.80	10.13	20.41	0.68
84_t1	4.40	1.65	8.68	1.18	0.94	8.90	7.45	17.55	1.05
89_t0	0.62	0.61	1.55	0.70	0.69	3.99	1.32	35.18	0.64
89_t1	1.36	1.50	4.53	1.64	1.37	3.60	10.86	9.37	1.64

	28	29	30	31	32	33	34
201_t0	3.80	7.14	1.38	1.32	2.11	1.11	1.20
201_t1	6.48	10.42	0.93	0.65	1.18	0.76	1.00
27_t0	2.39	16.49	1.00	1.15	0.96	0.96	1.01

	28	29	30	31	32	33	34
27_t1	2.53	17.99	1.22	2.27	1.35	1.08	1.38
28_t0	2.08	22.86	1.51	1.90	1.43	1.29	1.42
28_t1	2.62	13.66	0.73	1.26	1.27	0.80	1.25
31_t0	3.03	9.34	2.91	0.58	2.22	0.71	0.87
31_t1	2.60	4.43	0.65	0.29	3.17	0.30	2.88
32_t0	1.20	17.53	1.06	0.82	1.13	0.63	0.80
32_t1	1.69	13.54	0.89	0.83	2.25	0.58	0.77
33_t0	3.40	21.85	1.53	1.14	0.99	0.81	0.89
33_t1	4.73	19.77	0.77	1.53	1.00	0.99	0.52
34_t0	7.68	1.53	0.83	0.87	3.51	1.12	0.89
34_t1	9.71	7.57	1.35	1.19	1.28	0.89	1.14
35_t0	1.57	22.55	1.13	3.06	0.92	0.84	1.38
35_t1	1.57	24.34	0.98	3.92	1.04	0.70	0.85
37_t0	2.02	14.57	1.14	6.27	1.28	0.38	0.44
37_t1	1.65	16.30	1.38	1.95	1.22	0.41	0.51
59_t0	8.10	6.58	1.03	0.34	6.04	0.34	1.42
59_t1	4.75	12.90	1.12	1.64	1.74	0.65	0.64
63_t0	1.91	15.24	0.41	1.66	0.92	6.41	1.12
63_t1	3.16	4.63	0.79	4.13	3.80	0.50	0.72
65_t0	3.10	24.28	2.82	1.61	0.84	0.55	0.45
65_t1	1.95	33.97	1.48	1.35	1.15	0.86	0.83
66_t0	5.88	16.97	3.28	1.00	2.82	0.45	1.80
66_t1	3.74	8.34	1.64	1.64	3.03	1.79	1.40
78_t0	7.58	4.12	0.72	0.59	8.38	0.64	0.73
78_t1	3.24	7.82	1.75	1.61	2.00	1.97	1.66
80_t0	4.74	7.05	2.25	0.24	7.26	0.25	1.48
80_t1	4.20	19.10	0.72	1.02	3.66	0.68	0.85
82_t0	5.05	14.48	2.67	1.54	1.52	1.66	1.31
82_t1	2.35	22.55	1.51	1.11	1.19	4.55	0.99
83_t0	7.24	21.72	0.57	2.79	2.13	0.64	3.28

	28	29	30	31	32	33	34
83_t1	4.64	4.47	0.43	4.01	2.85	0.66	3.45
84_t0	2.39	1.38	0.79	1.60	2.22	0.77	0.99
84_t1	2.16	2.07	1.22	1.83	2.80	0.96	1.08
89_t0	0.67	30.36	0.83	3.43	0.62	0.82	0.77
89_t1	2.24	10.52	1.46	1.18	2.03	4.44	1.39

Healthy subjects for comparative study:

	1	2	3	4	5	6	7	8	9
HS1	0.2521	0.3476	1.7689	0.3321	0.1684	0.9519	0.7811	0.7292	0.1893
HS2	0.2497	0.8499	1.8122	0.3317	0.2029	1.5475	2.1279	0.259	0.1432
HS3	0.1481	0.2367	2.641	0.463	0.1004	0.977	3.7271	0.3177	0.12
HS4	0.5366	0.7253	0.4023	0.282	0.5489	0.3508	0.3071	0.3183	0.2967
HS5	0.361	0.3554	1.2503	0.2259	0.1812	0.6889	0.3366	0.2928	0.1719
HS6	0.6199	1.1297	0.7183	0.4699	0.4301	1.0287	1.6489	0.5683	0.4683
HS7	0.2165	0.3803	2.8645	0.2875	0.1758	0.4595	1.7546	0.218	0.2022
HS8	0.5103	1.9707	1.409	0.4938	0.3579	0.5426	1.5386	0.5449	0.3182
HS9	0.8328	2.092	1.3674	1.8255	0.6051	0.8673	1.909	0.59	0.6399
HS10	0.502	0.776	1.6018	0.3275	0.227	1.07	2.0915	0.4672	0.2516
HS11	0.2895	0.4331	0.3081	1.0653	0.325	1.0696	0.6626	0.2885	0.4387
HS12	0.391	0.4995	1.4856	0.2112	0.3192	1.5638	1.1797	0.6707	0.2383
HS13	0.6208	1.2139	1.198	0.5039	0.4577	1.1782	0.6271	0.5502	0.7499

	1	2	3	4	5	6	7	8	9
HS14	0.3324	0.8668	1.6517	0.3004	0.1995	0.8103	1.7092	0.3974	0.2735
HS15	0.2994	0.8356	0.7249	0.3	0.224	1.338	0.7324	0.402	0.2622
HS16	0.2871	0.2741	1.0933	0.1752	0.1337	1.9443	1.3313	0.4646	0.0997
HS17	0.2413	0.4346	0.9704	0.2094	0.1482	0.4151	0.3109	0.4107	0.1789
HS18	0.2954	0.5917	0.5008	0.1716	0.1739	0.3342	1.066	0.2559	0.23
HS19	0.8889	0.793	0.5666	0.3625	0.2789	0.6211	0.3928	0.7	0.3588
HS20	0.6405	0.8843	0.5672	0.3369	0.4888	0.5861	0.3193	0.38	0.3424
HS21	0.3237	0.619	0.8644	0.404	0.2327	0.6395	0.3139	0.4742	0.2664
HS22	0.2032	0.3795	2.1908	0.7713	0.419	0.3313	1.7752	0.3488	0.1677
HS23	0.6205	0.6325	0.3997	0.2381	0.2703	0.3913	0.4049	0.4849	0.294
HS24	0.6155	0.8252	0.5691	0.3075	0.3784	0.4741	0.3637	0.4922	0.5639

	10	11	12	13	14	15	16	17	18
HS1	0.406	2.5407	21.399	0.7386	0.1637	2.3453	15.975	7.2115	0.7691
HS2	0.4094	3.6887	25.529	0.5557	0.5536	2.409	7.3833	3.177	0.1811
HS3	0.2192	1.4882	13.812	0.4257	0.0976	2.1137	23.282	4.1006	0.1573
HS4	0.6393	0.6868	26.183	0.5543	0.2832	2.7427	1.0824	22.523	0.4247
HS5	0.4518	1.8647	19.933	0.5085	0.1812	1.9736	15.647	12.007	0.2114
HS6	0.8317	1.1987	35.3	0.7597	0.4414	2.1454	4.661	6.0275	0.5392

	10	11	12	13	14	15	16	17	18
HS7	0.3502	0.7964	11.414	0.4492	0.2727	1.1521	32.629	5.4433	0.1791
HS8	0.5457	1.6318	32.131	0.5694	0.1867	2.8843	4.739	14.405	0.3275
HS9	2.7625	1.0323	20.228	2.05	2.4394	2.5246	2.1549	12.464	1.9064
HS10	0.374	3.2136	24.126	0.7056	0.2772	3.0256	12.323	5.6171	0.2437
HS11	0.7427	1.0226	2.4654	0.5245	0.7797	14.147	3.7081	19.803	0.2194
HS12	1.8617	1.8946	8.9693	0.5047	0.3001	3.6787	8.0279	7.6682	0.3024
HS13	0.5078	1.4213	25.608	1.53	0.3695	3.4775	6.6021	13.326	0.4939
HS14	0.4438	2.9676	24.328	0.7337	0.2421	2.9674	13.171	6.914	0.2542
HS15	0.9126	1.3143	9.2998	0.7568	0.2538	12.246	7.2053	20.44	0.2803
HS16	1.7545	2.3931	15.481	0.5456	0.1038	5.8428	12.04	8.3541	0.1975
HS17	0.5099	1.4528	21.249	0.5546	0.1865	2.4566	18.202	13.35	0.2003
HS18	0.3614	0.5274	10.663	0.6747	0.1868	1.6567	45.246	5.9097	0.1852
HS19	0.506	1.1394	29.805	0.7977	0.3654	4.0422	4.8995	17.575	0.3516
HS20	0.6299	0.9753	26.474	0.9464	0.253	2.9776	6.6293	17.721	1.1107
HS21	0.5828	1.3038	19.343	0.7011	0.2616	2.6031	20.873	14.313	0.2576
HS22	0.2457	1.086	10.411	0.5143	0.1418	1.6264	41.146	4.2271	0.3057
HS23	0.6236	0.9823	29.378	0.7208	0.2242	4.0409	5.5988	17.761	0.2857
HS24	0.6203	1.6284	30.902	0.9004	0.2592	3.2791	7.3661	12.812	0.5389

	19	20	21	22	23	24	25	26	27
HS1	0.2537	0.2443	8.0839	1.9839	4.2084	0.2174	0.2624	5.6287	1.0538
HS2	0.2141	0.5139	9.5881	3.6509	8.3965	0.8214	0.2799	3.6918	4.044
HS3	0.1299	0.184	9.0344	1.4048	6.2385	0.168	0.1814	9.4537	0.9364
HS4	0.523	0.3593	1.4043	0.6577	1.7167	0.5721	1.7468	5.6698	2.5911
HS5	0.2843	0.3557	5.5402	1.6529	3.5693	0.3094	0.3489	6.9275	1.4187
HS6	0.7789	0.597	6.4987	1.5355	2.8471	1.2536	0.5445	1.1895	3.6281
HS7	0.1951	0.449	6.4494	1.1473	4.0956	0.2473	0.2079	11.084	0.9354
HS8	0.3925	0.2782	3.484	0.5802	2.0265	0.6297	0.4355	3.678	2.0173
HS9	0.7739	1.9068	3.565	1.0171	1.7422	2.1033	0.6909	1.4832	5.9189
HS10	0.4998	0.4865	9.1618	3.2401	4.7489	1.1163	0.4055	2.4861	4.6008
HS11	2.499	1.3053	2.863	0.754	3.8199	3.6089	2.4525	1.4023	1.4299
HS12	1.5311	0.2556	6.0289	4.665	7.8084	0.4348	0.4059	5.1036	0.8376
HS13	0.4587	0.4328	4.7667	1.1298	3.7577	1.2639	0.4955	1.5559	4.9212
HS14	0.3008	0.4037	8.6543	3.0806	4.1709	1.139	0.3224	2.4177	4.4624
HS15	0.5975	0.311	4.0307	1.0914	2.7062	0.5315	0.5375	2.9034	0.8112
HS16	0.1296	0.1347	9.3251	3.9004	5.3727	0.24	0.2993	2.7654	0.6537
HS17	0.4281	0.2115	4.5582	1.0694	2.5029	0.3673	0.2491	5.5642	1.3994
HS18	0.2751	0.296	4.0184	0.8811	0.7889	0.4404	0.4497	9.2985	1.376

	19	20	21	22	23	24	25	26	27
HS19	0.5617	0.3257	3.7717	0.5721	1.388	0.5417	0.4059	6.2007	2.3785
HS20	0.4794	1.0026	3.9807	0.7132	0.742	1.6297	0.4478	0.7457	4.9458
HS21	0.3432	0.2728	3.9736	0.946	1.9626	0.3839	0.3444	5.3697	1.5598
HS22	0.2129	0.236	5.8925	1.3306	3.7417	0.3596	0.2048	8.491	0.9902
HS23	0.4352	0.2754	3.3818	0.5982	1.2855	0.5284	0.3557	6.517	2.3443
HS24	0.481	0.298	3.3627	0.7797	1.8908	2.1207	0.4536	0.8676	4.6866

	28	29	30	31	32	33	34
HS1	4.150179	9.278325	0.82764	1.882997	0.548024	0.234012	0.27025
HS2	3.869892	4.931696	2.478049	0.4344	0.669452	0.256973	0.294333
HS3	2.111133	5.44756	1.637268	3.20485	0.658592	0.202208	0.13701
HS4	0.784369	15.13325	3.330237	2.836168	0.474948	0.377409	0.402132
HS5	3.510455	12.15612	0.970873	3.083745	0.453074	0.090299	0.252901
HS6	2.436507	5.910112	4.612751	1.130444	0.887057	0.534239	0.638397
HS7	1.328906	6.969357	1.37832	2.323454	0.392552	0.220732	0.240548
HS8	1.029425	11.40181	3.395659	2.548808	0.481189	0.12971	0.277728
HS9	2.897009	8.057857	3.426319	1.795201	1.501705	0.443831	0.839544
HS10	2.978875	4.712036	3.139784	0.536465	0.627043	0.376926	0.365966
HS11	1.703096	20.63053	0.274607	0.8626	1.045756	2.789146	0.946347

	28	29	30	31	32	33	34
HS12	7.388228	11.46457	0.763486	0.800529	2.490298	0.448324	1.229064
HS13	2.585649	8.435593	2.964544	1.891725	0.832984	0.856676	0.671091
HS14	3.170746	4.879374	3.023485	0.48939	0.420601	0.287663	0.524289
HS15	2.689284	19.70278	0.401608	0.877934	1.162587	0.29218	0.288695
HS16	5.008319	11.8164	0.603528	0.566201	1.690401	0.151447	0.181522
HS17	3.734015	11.4004	0.90549	3.20276	0.364324	0.17914	0.189535
HS18	0.926931	6.858228	0.563526	1.518335	0.273798	0.275126	0.224017
HS19	1.122669	10.4729	2.700028	2.123529	0.441868	0.335099	0.313154
HS20	2.328325	10.67527	4.318832	2.497872	0.43233	0.460428	0.352506
HS21	3.37187	10.46361	0.970274	2.687751	0.443748	0.27732	0.286154
HS22	1.381211	5.683368	0.814678	1.928098	0.391056	0.431337	0.153139
HS23	1.306624	11.50628	2.614276	2.401733	0.427142	0.299445	0.318814
HS24	2.551476	9.608119	4.708712	2.358174	0.310065	0.360346	0.321425

Annex 4. Bacterial groups detected by FISH in the culture broth recovered from each stage (Vessel 1, Vessel 2 and Vessel 3) of the colonic model before (SS1) and after (SS2) the daily administration of the four dietary flours.

Modifications at a confidence level of 95% ($P < 0.05$) are represented as underlined or italic in case of significant increase or decrease, respectively, between SS1 and SS2.

	Nutriwheat (NW)					
	SS1			**SS2**		
	Vessel 1	**Vessel 2**	**Vessel 3**	**Vessel 1**	**Vessel 2**	**Vessel 3**
FPrau655	8.79 ± 0.07	8.82 ± 0.11	8.46 ± 0.11	8.66 ± 0.13	8.11 ± 0.16	7.75 ± 0.13
Bif164	7.38 ± 0.11	7.26 ± 0.16	7.28 ± 0.14	7.70 ± 0.04	**7.61 ± 0.14**	7.45 ± 0.08
Lab158	7.70 ± 0.16	7.79 ± 0.18	7.85 ± 0.17	**8.23 ± 0.26**	7.97 ± 0.18	7.72 ± 0.08
Ere432	8.67 ± 0.09	8.71 ± 0.07	8.58 ± 0.11	8.71 ± 0.13	8.70 ± 0.15	8.56 ± 0.10
Ato291	8.63 ± 0.12	8.53 ± 0.13	8.42 ± 0.12	8.69 ± 0.08	8.63 ± 0.12	8.39 ± 0.17
Rre534	7.99 ± 0.21	7.77 ± 0.07	7.87 ± 0.07	7.91 ± 0.28	7.57 ± 0.15	7.51 ± 0.16
EUB338	9.75 ± 0.12	9.49 ± 0.07	9.38 ± 0.09	9.57 ± 0.07	9.43 ± 0.10	9.05 ± 0.06
Bac303	8.58 ± 0.26	8.94 ± 0.07	8.41 ± 0.19	8.79 ± 0.22	8.94 ± 0.21	8.82 ± 0.11
RbroRfla	8.92 ± 0.06	8.55 ± 0.19	8.30 ± 0.11	***8.38 ± 0.15***	***7.87 ± 0.08***	***7.53 ± 0.09***
DSV567	6.49 ± 0.43	7.03 ± 0.39	6.45 ± 0.29	7.22 ± 0.16	7.50 ± 0.17	**7.83 ± 0.27**
Prop853	8.97 ± 0.14	8.79 ± 0.13	8.60 ± 0.11	8.82 ± 0.09	8.76 ± 0.13	8.65 ±0.08
Chis150	8.21 ± 0.09	8.13 ± 0.16	7.93 ± 0.23	8.15 ± 0.24	7.66 ± 0.31	7.67 ± 0.35

	Wholegrain Rye (WG)					
	SS1			SS2		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
FPrau655	8.71 ± 0.09	8.58 ± 0.07	8.24 ± 0.13	8.36 ± 0.38	8.63 ± 0.14	8.61 ± 0.15
Bif164	7.46 ± 0.13	7.30 ± 0.12	7.26 ± 0.14	**8.54 ± 0.23**	**8.65 ± 0.22**	**8.71 ± 0.25**
Lab158	7.81 ± 0.29	7.64 ± 0.24	7.73 ± 0.19	**8.56 ± 0.19**	**8.73 ±0.23**	**8.52 ± 0.23**
Ere432	8.68 ± 0.11	8.80 ± 0.10	8.60 ± 0.14	8.04 ± 0.38	8.84 ± 0.15	8.83 ± 0.13
Ato291	8.58 ± 0.17	8.50 ± 0.13	8.55 ± 0.13	8.88 ± 0.12	8.77 ± 0.11	8.72 ± 0.13
Rre534	7.88 ± 0.17	7.91 ± 0.14	7.70 ± 0.21	***7.26 ± 0.22***	7.77 ± 0.16	7.64 ± 0.08
EUB338	9.67 ± 0.08	9.41 ± 0.06	9.17 ± 0.17	9.61 ± 0.15	9.55 ± 0.07	9.43 ± 0.09
Bac303	8.64 ± 0.27	8.92 ± 0.17	8.60 ± 0.16	8.78 ± 0.24	9.14 ± 0.09	8.85 ± 0.11
RbroRfla	8.53 ± 0.21	8.44 ± 0.16	8.28 ± 0.18	8.29 ± 0.25	8.34 ± 0.15	7.94 ± 0.08
DSV567	6.61 ± 0.40	6.66 ± 0.26	6.65 ± 0.15	**7.38 ± 0.37**	**7.49 ± 0.29**	**7.78 ± 0.28**
Prop853	8.80 ± 0.15	8.73 ± 0.12	8.73 ± 0.15	8.80 ± 0.17	8.95 ± 0.08	8.74 ± 0.11
Chis150	8.18 ± 0.12	8.20 ± 0.09	8.21 ± 0.17	7.27 ± 0.37	8.35 ± 0.28	8.40 ± 0.20

	Barley milled grains (BMG)					
	SS1			SS2		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
FPrau655	8.96 ± 0.15	8.79 ± 0.17	8.68 ± 0.12	8.79 ± 0.28	8.70 ± 0.06	8.44 ± 0.05
Bif164	8.72 ± 0.09	8.65 ± 0.07	8.52 ±0.08	8.74 ± 0.50	8.96 ±0.11	8.67 ±0.26
Lab158	8.82 ± 0.07	8.68 ± 0.07	8.52 ± 0.05	8.63 ± 0.14	8.67 ± 0.04	8.50 ± 0.03
Ere432	8.96 ± 0.09	8.88 ± 0.17	8.94 ± 0.06	8.58 ± 0.29	8.80 ± 0.06	***8.75 ± 0.06***
Ato291	8.72 ± 0.04	8.82 ± 0.03	8.83 ± 0.02	8.87 ± 0.08	8.72 ± 0.07	8.60 ± 0.09
Rre534	8.30 ± 0.16	8.12 ± 0.08	8.14 ± 0.13	7.82 ± 0.38	***7.77 ± 0.15***	***7.39 ± 0.10***
EUB338	9.38 ± 0.44	9.66 ± 0.15	9.25 ± 0.10	9.51 ± 0.17	9.37 ± 0.11	9.23 ± 0.12
Bac303	8.34 ± 0.29	8.59 ± 0.13	8.47 ±0.17	8.69 ± 0.25	8.93 ± 0.09	8.47 ±0.14
RbroRfla	7.93 ± 0.40	8.65 ± 0.11	8.50 ± 0.18	7.64 ± 0.63	7.59 ± 0.60	8.33 ± 0.09
DSV567	6.77 ± 0.11	6.87 ± 0.09	7.04 ± 0.09	**7.41 ± 0.19**	**7.63 ± 0.18**	7.54 ± 0.21
Prop853	8.65 ± 0.16	8.80 ± 0.12	8.80 ± 0.13	8.80 ± 0.05	8.81 ±0.09	8.56 ±0.09
Chis150	8.53 ± 0.07	8.57 ± 0.10	8.44 ± 0.14	8.36 ± 0.17	8.64 ± 0.08	8.49 ± 0.15

	Pulses (PF)					
	SS1			SS2		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
FPrau655	8.86 ± 0.09	8.75 ± 0.08	8.65 ± 0.13	8.93 ± 0.06	***8.50 ± 0.05***	***8.19 ± 0.08***
Bif164	8.05 ± 0.45	8.57 ± 0.08	8.25 ± 0.13	8.41 ± 0.19	8.89 ± 0.19	8.78 ± 0.08
Lab158	8.73 ± 0.09	8.63 ± 0.07	8.46 ± 0.14	8.64 ±0.04	8.61 ± 0.04	8.48 ± 0.05
Ere432	8.99 ± 0.06	8.94 ±0.06	8.86 ± 0.11	***8.60 ±0.04***	8.77 ± 0.05	8.70 ± 0.05
Ato291	8.74 ± 0.05	8.79 ± 0.06	8.76 ± 0.06	***8.87 ± 0.03***	8.93 ±0.09	8.80 ±0.06
Rre534	8.61 ± 0.13	8.23 ±0.11	8.06 ± 0.14	***7.52 ± 0.32***	***7.55 ± 0.14***	***7.46 ± 0.19***
EUB338	9.67 ± 0.11	9.26 ±0.08	9.26 ± 0.12	9.76 ± 0.07	9.48 ± 0.12	9.35 ± 0.07
Bac303	8.32 ± 0.41	8.63 ±0.13	8.53 ± 0.19	**9.45 ± 0.04**	**9.09 ± 0.05**	**8.95 ± 0.07**
RbroRfla	7.50 ± 0.55	8.39 ± 0.11	8.18 ± 0.11	6.25 ± 0.05	8.50 ± 0.06	**8.51 ± 0.07**
DSV567	7.38 ± 0.32	7.36 ± 0.27	6.88 ±0.44	7.12 ± 0.38	6.89 ± 0.35	6.72 ± 0.34
Prop853	8.79 ±0.10	8.83 ± 0.09	8.66 ±0.04	8.79 ±0.05	***8.64 ± 0.04***	8.55 ±0.05
Chis150	8.60 ± 0.08	8.21 ± 0.11	8.14 ±0.13	8.19±0.15	8.41 ±0.08	8.47± 0.09

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