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Probiotics for the prevention/treatment of human diseases and ecological study of the intestinal microbiota

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Abstract

The interest in the intestinal microbiota of humans has increased in the last 20 years both by clinicians and biologists and significant advances have been achieved with regard to its composition and functions. The gut microbiota is involved in a range of complex interactions with the host and strongly contributes to the maintenance of the host's health status. For this reason, alterations in the gut microbiota have been involved in the pathogenesis of some diseases or in their progression. Therefore, several studies have been focused on the manipulation of the gut microbiota composition in order to promote the health of the host. Probiotics, with their long history of safety and effectiveness against harmful microorganisms, are a strategy not only to maintain or restore the correct balance in the microbial population of the intestinal tract, but also to prevent or treat a range of disease conditions.

The aim of the work proposed in this thesis was to explore the possibility of probiotic supplementation for the prevention/treatment of diseases of different origin in humans and the related study of the intestinal microbial environment.

The first step was to review the studies concerning the use of *Bifidobacterium breve* as probiotic in paediatric diseases. Secondly, the effectiveness of a probiotic formulation consisting of two strains of *B. breve* was evaluated in paediatric subjects in order to prevent or treat some gastrointestinal tract-related disorders including coeliac disease. Moreover, the same probiotic formulation was assessed in obese paediatric patients to evaluate the effect on the progression of the disease.

As the emerging role of gut microbiota in neurological diseases, a brunch of this work focused on the evaluation of intestinal microbial environment in amyotrophic lateral sclerosis patients compared to healthy controls and on the effects of a probiotic administration on the disease progression.

The last part of this work was dedicated to the investigation not only of gut bacterial community of preterm infants, but also on bacteriophage one considering that emerging evidences have shown that viruses seem to play a fundamental role in shaping the gut bacterial composition.

The technical approach in these studies consisted mainly to correlate microbial data, obtained with the integrated approach of qPCR plus next generation sequencing, and clinical data collected by the medical staff.

The results obtained in this thesis firstly evidenced differences in gut microbial composition of healthy controls compared to that of diseased subjects in coeliac and amyotrophic lateral sclerosis patients. The probiotic approach was effective in restoring the microbial composition in the former, whereas, in the latter, the influence was focused only on some microbial groups. Secondly, the probiotic intervention was found effective in improving the glyco-insulinemic profile in obese children and in preventing gastrointestinal disorders in healthy newborns. Thirdly, the study of the bacterial and phage composition in preterm infants suggested a transkingdom interplay between bacteria and viruses with a reciprocal influence on their composition.

Keywords: gut microbiota, probiotics, human, disease, health, paediatrics, infants, coeliac disease, obesity, ALS, preterm

TABLE OF CONTENTS

INTRODUCTION

1. GUT MICROBIOTA

1.1 The power of the Superorganism

The human microbiota is the whole population of microorganisms that colonize the human body, including bacteria, fungi, archaea, viruses and protozoans. It establishes a symbiotic relationship with the host contributing to metabolic activities. Its genome, which is more than 100 times larger than the human genome, is defined "microbiome" (Del Chierico et al., 2012). The human microbiota and the host constitute a "Superorganism", since the global metabolism is the result of the interactions between two integrated genomes, the human genome, genetically inherited from parents, and the human microbiome, acquired from the environment after birth (Lederberg, 2000). The Human Microbiome Project (HMP) has been carried out over ten years in two phases to provide resources, methods, and discoveries about interactions between human health and their microbiomes.

As the majority of human microbiota resides in the gastrointestinal tract, in the last twenty years the gut microbiota has drawn the interest of the scientific community. In contrast to the human genome, the gut microbiome is more flexible and can be modulated by external factors. Alteration of the normal composition of gut microbiota can affect the host metabolism since it consists of functions encoded by both human genome and microbiome. These two genomes have to work in harmony to maintain the health status of the host (Jia et al., 2008). This superorganism concept constitutes a new and complete view on human organisms for managing health and wellness (Zhao & Shen, 2010).

1.2 Normal gut microbiota composition

In the human gastrointestinal tract there are substantial differences in terms of composition and amount of microbes ranging from 10^1 cells per gram of contents in the oesophagus and stomach to 10^{12} cells per gram of contents in the colon and distant gut (O'Hara & Shanahan, 2006). *Streptococcus* is the predominant genus in the distal oesophagus, duodenum and jejunum (Pei et al., 2004; Justesen et al., 1984). The genus *Helicobacter* is dominant in the stomach (Andersson et al., 2008).

The large intestine hosts more than the 70% of all human microbiota. The predominant phyla in a healthy gut microbiota are Firmicutes and Bacteroidetes, followed by Actinobacteria and Verrucomicrobia. As traditionally beneficial bacteria belong to Firmicutes and potentially harmful bacteria to Bacteroidetes, the Firmicutes/Bacteroidetes (F/B) ratio is considered a parameter to determine a dysbiosis condition (Ley e al. 2006).

Some bacterial groups resulted more abundant in the lumen, i.e. *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Lactobacillus*, *Ruminococcus*; the genera that are also detected in the mucus layer are *Clostridium*, *Lactobacillus*, *Ruminococcus* and *Akkermansia* (Swidsinski et al., 2005).

The MetaHit Consortium proposed models based on different microbial clusterizations, which are stable over geography and gender, but respond in a different way to external perturbations, such as diet and drugs; these clusters are named "enterotypes" (Arumugam et al. 2011). These have been classified into 3 groups (Hollister et al., 2014). Enterotype 1, which has high abundance of

Bacteroides, is characterized by a saccharolytic potential, extrapolating energy from dietary carbohydrates; enterotype 2, which has high abundance of *Prevotella*, is characterized by bacterial degraders of mucin glycoproteins that constitute the gut mucosal layer; enterotype 3, which has high abundance of *Ruminococcus*, is also associated to mucin degradation and membrane transport of carbohydrates.
Microbiologists' attention has also been focused on bacteriophages, which, living at the expense of bacteria, establishing a "predator-prey" relationship and being vehicles of genetic transfer, could have an important role in shaping the biodiversity of the gut ecosystem. The first metagenomic analysis of an uncultured viral community from human faeces using partial shotgun sequencing revealed a large diversity of phages in gut microbiota; the community contained about 1200 virus genotypes and the recognizable viruses were mostly siphophages (Breitbart et al 2003). In healthy adults, bacteriophages' community consists mostly of members of the order Caudovirales and family Microviridae. These bacteriophages typically maintain a stable status over time (Reyes et al., 2010; Minot et a., 2011). Although there are some evidences that shifts in the enteric bacteriophage community composition are associated with Crohn's disease and ulcerative colitis (Norman et al., 2015), the role of bacteriophages in human disorders has to be still elucidated.

1.3 Factors shaping the gut microbiota

1.3.1 Age

The gut microbial colonization starts soon after birth, but there is emerging evidence that the infant gut is colonized even *in utero* (Dominguez-Bello et al., 2010). In support of this, there are some studies that detected bacteria in the placenta (Aagaard et al., 2014; Rodriguez e al., 2015). Moreover, Gosalbes et al. (2013) reported the main bacterial genera present in the meconium: *Escherichia-Shigella*, *Enterococcus*, *Leuconostoc*, *Lactococcus* and *Streptococcus*.
However, the first external factor that mostly influences the gut colonization is the mode of the delivery. Guts of infants' born by vaginal delivery are mostly populated by bacteria deriving from the maternal vaginal microbiota, such as members belonging to *Lactobacillus* and *Preovotella* genera (Makie et al., 1999). In those born by caesarean section, the intestinal colonization starts from microorganisms deriving from the maternal skin microbiota, as members belonging to *Streptococcus*, *Corynebacterium* and *Propionibacterium* genera (Dominguez-Bello et al., 2010; Makie et al. 1999); in addition, they are characterized by a depletion in *Bacteroides* colonization in favour of facultative anaerobes, such as *Clostridium* species (Jakobsson e al., 2014; Salminem et al., 2004).
Since the positive oxidation/reduction potential of the neonates' intestine at birth, facultative anaerobes bacteria remain predominant during the first few days of life; among them, *Staphylococcus* spp., Enterobacteriaceae, and *Streptococcus* spp. are most commonly isolated from newborn faeces. Then, strict anaerobes, such as members of the *Bifidobacterium*, *Bacteroides*, and *Clostridium* genera, become predominant (Di Gioia et al., 2014).
The gut microbiota biodiversity increases rapidly during the first years of life towards an adult gut microbiota setting. During childhood and in teens, hormonal and sexual maturation, social behaviour, adult-like diet continue to affect, although to a lesser extent, the gut microbiota (Putignani et al., 2014).

In adulthood, the gut microbiota composition is relatively stable, but it is still subjected to perturbation by life events (Dethlefsen et al., 2011).
The microbial diversity and functionality decrease during the senescence. In subjects aged over 65, the microbial community shifts, with an increased abundance of Bacteroidetes at phylum level and *Clostridium* cluster IV at genus level (Claesson et al., 2011). The centenarian microbiota also exhibits alterations, such as an increase of facultative anaerobes and a decrease of butyrate producers (Biagi et al., 2010). In addition, some metabolic functions result compromised, particularly the short-chain fatty acid (SCFA) production and amylolysis activity are reduced, although proteolytic activity is increased (Biagi et al., 2013).

1.3.2 Diet

Diet exerts a large effect on the gut microbiota. After the mode of delivery, the earliest influence on gut microbiota depends on the type of feeding. Many studies have detected marked differences in the intestinal microbiota composition of breast- and formula-fed infants. The bioactive compounds present in breast milk contribute to the nutrient digestion and adsorption, immune protection and anti-microbial defence (Albenberg et al., 2014; Brown et al., 2013). Among these, human milk oligosaccharides (HMOs) provide a selective growth for bifidobacteria (Zivkovic et al., 2011); consequently, the abundance of *Bifidobacterium* spp. is much higher in breast-fed infants compared to formula-fed ones (Yu et al., 2013; Bezirtzoglou et al., 2011). In addition, these bacteria are also able to ferment dietary oligosaccharides and modulate the expression of IgA and IgE by the host immune system (Ouwehand et al., 2002). Even though *Bacteroides* can also digest HMOs, the abundance of *Bifidobacterium* remains higher in breast-fed infants, thus showing a competition between these two organisms in favour of *Bifidobacterium* (Jandhyala et al., 2015).
Moreover, undernourished infants, having an immature gut microbiota, show dysbiosis and, particularly, a higher amount of Enterobacteriaceae (Kau et al., 2015). A study carried out by De Filippo et al. (2010) demonstrated that rural African children, with a diet dominated by starch, fibers and plant polysaccharides, revealed a gut microbiota that is abundant in Actinobacteria (10.1%) and Bacteroidetes (57.7%). On the other side of the world, in European children, whose diet is rich in sugar, starch and animal proteins, these bacterial phyla resulted reduced to 6.7% and 22.4% respectively. In addition, some SCFAs producers, as *Prevotella*, resulted exclusive to the African children microbiota.
Diet continues to be one of the most influent factor that affects intestinal microbial composition also during adulthood. Commonly, the intake of dietary fibre impacts on the microbiota diversity and the dominant species. A diet rich in fruits, vegetables and fibres is linked to a high richness and diversity of gut microbiota, as well high levels of bacteria able to metabolize insoluble carbohydrates, as *Ruminococcus*, *Roseburia* and *Eubacterium* (Walker et al., 2011). An interesting correlation between the consumption of fruits, vegetables and fibres within a Mediterranean diet and high levels of SCFAs has been found (De Filippis et al., 2015).
Different dietary pattern can also affect the gut microbiota by influencing the production of specific metabolites. Diet rich in animal proteins and fat has been strongly associated to a consistent production of trimethylamine N-oxide (TMAO) from L-carnitine and phosphotidylcholin; particularly, a significantly correlation was found between TMAO and L-*Ruminococcus* (De Filippis et al., 2015). Noticeably, high levels of TMAO in blood, negatively affecting the

cholesterol metabolism, are associated with an increased risk of cardiovascular disorders (Wilson et al., 2014).
Intestinal microorganisms are able to metabolize some dietary fats, even if most of them are absorbed in the small intestine (Scott et al., 2013). Conjugated linoleic acid (CLA) has been naturally found in red meat and dairy products (O'Shea et al., 2004) and can also be produced by the intestinal microbes (McIntosh et al., 2009; Gorissen et al., 2010). The presence of CLA and their isomers has been correlated with some bacterial groups. A study carried out by Devillard et al. (2009) evidenced high levels of rumenic acid, belonging to CLA, in faecal samples of individuals showing a prevalence of Bacteroidets in gut microbiota.

1.3.3 Antibiotics

Antibiotic treatments result in several short and long-term implications in gut microbial balance, including reduced richness and diversity. Particularly, the impact of antibiotics on the intestinal microbiota of newborns results in the reduction of the major anaerobic bacteria with a consequent overgrowth of enterococci and Enterobacteriaceae (Fouhy et al., 2012). Moreover, antibiotic treatments in early-life may cause a consistent depletion of lactobacilli and bifidobacteria counts (Bennet et al., 2002; Mangin et al., 2010).
Clindamiycin, clarithtromycin, metronidazole and ciproflaxin have an influence on the microbiota composition that depends on the duration of the treatment (Jernberg et al., 2007; Jakobsson et al., 2010; Dethlefsen et al., 2011). A clinical trial showed that a β-lactam therapy including ampicillin, sulbactam and cefazolin affects the gut microbial composition and metabolites involved in cellular functions (Ferrer et al., 2014). A study conducted by Ge et al., (2017) on mice models evidenced that microbiota depletion associated to antibiotic treatments affects secondary bile acid and serotonin metabolism in the colon, leading to a reduced intestinal peristalsis.

1.4 Role of the gut microbiota in health

The gut microbiota exerts a mutualistic relationship with the host providing substantial metabolic, immunological and protective functions for the host's health.

1.4.1 Metabolic function

The intestinal microbiota is able to exert a wide range of metabolic activities that have an important impact on host health.
Gut microorganisms mostly derive their nutrients and energy from dietary carbohydrates. *Bacteroides*, *Roseburia*, *Bifidobacterium*, *Fecalibacterium* and some Enterobacteriaceae members are able to ferment indigestible oligosaccharides producing SCFAs, as butyrate, propionate and acetate, which constitute energy sources for the host being adsorbed by the epithelial cells (Macfarlane et al., 2003; Sartor et al., 2008). SCFAs also play a protective role for the host: acetic acid has been demonstrated to modulate cell turnover reducing the risk of inflammatory disease (Hooper 2004; Comalada et al., 2006) and butyrate can prevent the accumulation of toxic by-products (Bourriaud et al., 2002).
The gut microbiota is also crucial for the synthesis of essential vitamins. Specifically, lactic acid bacteria play a key role in the production of vitamin B12, which cannot be synthetized by animals, plants or fungi (Martens et al., 2002). Moreover, it has been demonstrated that bifidobacteria are

main producers of folate, a vitamin involved in DNA synthesis and repair processes (Pompei et al., 2007). Further vitamins, which are synthetized by gut microbiota, include vitamin K, riboflavin, biotin, nicotinic acid, panthotenic acid, pyridoxine and thiamine (Hill, 1997).
The gut microbiota also possesses an efficient protein metabolizing function contributing to the human proteinases activities. L-histidine is converted to histamine by the bacterial enzyme histamine decarboxylase, encoded by the bacterial *hdcA* gene (Thomas et al., 2012); the glutamic acid is converted to γ-amino butyric acid (GABA) by the glutamate decarboxylases, encoded by the bacterial *gadB* gene (De Biase et al., 2012).
Members belonging to *Bacteroides* genus are able to produce CLAs, which are able to have antidiabetic, antiobesogenic, hypolipidemic and immunomodulatory properties (Baddini Feitoza et al., 2009; Devillard et al., 2007; Devillard et al., 2009). Some species, as *Bacteroides intestinalis*, *Bacteroides fragilis* and *Escherichia coli*, have to be shown to deconjugate and dehydrate the primary bile acids that are not reabsorbed to the ileum and to convert them into secondary bile acids (Fukiya et al., 2009). In addition, it has been proven that gut microbiota modulates host energy influencing serum levels of pyruvic acid, citric acid, fumaric acid and malic acid, which are considered indicator of a high energy metabolism (Velagapudi et al., 2010).
Intestinal microorganisms are also involved in the biotransformation of poliphenols assumed with the diet to active compounds that are then absorbed by the portal vein and transported to tissues and organs. One of this mechanisms is the conversion of inactive flavones to the aglycon equol, which possess antiandrogenic, hypolipidemic and neuroprotective effects (Marín et al., 2015).

1.4.2 Protective function

The intestinal microbiota maintains the integrity of the gut barrier and the structure of the intestinal tract. The attachment of pathogens to the surface of the intestinal epithelium is a crucial initial step for infection occurring. As a defence mechanism, the epithelium produces mucus and antimicrobial molecules to inhibit pathogen invasion. The gut microbiota contributes to antagonize these attacks through secretion of antimicrobial molecules, competition for nutrients and ecological sites and mucosal immune system stimulation (Jankowska et al., 2008).
Segmented filamentous bacteria (SFB), a class of anaerobic and *Clostridium*-related sporeforming, are intimately attached to the epithelial surface of the mammalian gastrointestinal tract and actively interact with immune system (Jepson et al., 1993; Schnupf et al., 2015). Germ free (GF) mice possess an extremely thin adherent colonic mucus layer, but, when exposed to bacterial products (peptidoglycan or LPS), the thickness of the adherent mucus layer is restored resembling that of normal mice. This may indicate a contribution of the gut microbiota to mucus production (Petersson et al., 2011).
In addition, SCFAs, especially acetate produced by bifidobacteria, act on the intestinal epithelium during a bacterial infection to inhibit the translocation of Shiga toxin that is produced by *E. coli* O157:H7 (Fukuda et al., 2011).
Moreover, colonic bacteria can control the proliferation and differentiation of epithelial cells, contributing to the cellular turn-over in the intestinal crypts (Hooper 2004).

1.4.3 Immunomodulation function

The coevolution of the immune system with the gut microbiota has led to the development of molecular and cellular mechanisms, which contribute to maintain a condition of homeostasis. Studies on GF mice demonstrated that the intestinal microbiota is a key contributor to the proper structuring of inductive sites where intestinal immune cells, as Peyer's patches, mesenteric lymph nodes, lymphoid follicles and colonic patches, are localized (Macpherson and Harris, 2004). Some other studies demonstrated that GF mice have an immature intestinal immune system, showing hypoplastic Peyer's patches, under-developed gut-associated lymphoid tissues (GALT) and reduced circulating CD4+ T and plasma cells (Hooper et al., 2012).

Members of the RegIII family of C-type lectins, which bind to peptidoglycans of bacterial wall and are expressed in Paneth cells and absorptive enterocytes, have bactericidal properties. Particularly, the expression of Reg IIIg/HIP-PAP, which has been documented to protect the host from foodborne enteropathogens, such as Listeria (Brandl et al., 2007; Sanos et al., 2009), is dependent on the gut microbiota (Cash et al., 2006).

The gut microbes, in combination with CD8+ T cells, cooperate to regulate the systemic number of plasmacytoids (pDCs), a subgroup of dendritic cells that constitute the preeminent antigen-processing and -presenting cells of the immune system (Fujiwara et al., 2008).

The gut microbiota is also able to interact with some classes of toll-like receptors (TLRs), expressed on different immune cells, which are involved in the immune response and can recognize microbial molecules. Specifically, TLR9 binding ligands derived from the microbiota, plays an important role in balancing T regulatory and T effector cell immune responses and in host defence for *Encephalitozoon cuniculi*, a microsporidian parasite that causes diarrheal, respiratory and neurological diseases in immunocompromised subjects (Hall et al., 2008). It has also been demonstrated that gut microbiota stimulates protective T cell responses to oral infection from *Toxoplasma gondii* through TLR 2, 4 and 9 (Benson et al., 2009).

The intestinal microbiota is essential in driving the differentiation of Treg cells, which produce anti-inflammatory cytokines, such as IL-10 and TGF-β. In addition, the microbiota, inducing T_H1 cell differentiation, is involved in the establishment of a proper balance between T_H1 and T_H2 immune responses, which is crucial for the development of the immune tolerance (McLoughlin, and Mills, 2011); if an appropriate immune tolerance is not established in early life and maintained throughout life, the this risk to develop inflammatory, autoimmune, and allergic diseases is high (McLoughlin, and Mills, 2011).

Moreover, the interaction between some commensal bacteria and enterocytes can down-regulate the excessive release of cytokines from immature intestinal cells induced by pathogens in early life, contributing to a correct activity of the innate immune response (Cencic and Langerholc, 2010).

Bacteroides fragilis, a member of the healthy human gut microbiota, expresses several capsular polysaccharides, including polysaccharide A (PSA), which has been shown important immunomodulatory functions in mouse models of colitis reducing the proinflammatory cytokines IL-23 and IL-17 levels and increasing the anti-inflammatory cytokine IL-10 levels (Mazmanian et al., 2008).

2. DYSBIOSIS IN DISEASES

It has been well established that a functional and balanced microbiota contributes to the health status of the host; on the other hand, some diseases are known to be associated with a decrease of beneficial bacteria and increase of harmful ones in the gut microbiota. The number and typology of diseases for which an unbalanced gut microbial composition has been observed as a causative or consequential agent is increasing.

2.1 Dysbiosis in gastrointestinal tract-related disorders

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the colon and small intestine; Crohn's disease (CD) and ulcerative colitis (UC) constitute the most prevalent forms of IBD (Talley and O'Conor, 2013). A reduction of Firmicutes and an increase of Bacteroidetes at the phylum level and a general increase of facultative anaerobes, such as Enterobacteriaceae, have been documented in the gut microbiota of IBD patients (Hansen et al., 2010). Particularly, a reduction of a major member of Firmicutes, *Faecalibacterium prausnitzii*, has been associated to a higher risk of postoperative recurrence of ileal compliances in CD patients (Sokol et al., 2008). Other studies evidenced the prevalence of *E. coli* and *Mycobacterium paratuberculosis* in gut microbiota of CD patients suggesting an implication of these species in the pathogenesis of the disease (Darfeuille-Michaud et al., 2004; Rosenfeld and Bressler, 2010). A work focused on a cohort of newly diagnosed paediatric CD patients found increased abundance of Enterobacteriaceae, Pasteurellaceae, Veillonellaceae, and Fusobacteriaceae, and decreased abundance in Erysipelotrichales, Bacteroidales, and Clostridiales compared to healthy controls (Gevers et al., 2014).

UC patients have been showed a lower biodiversity in gut microbiota as well a reduction of anaerobic bacteria, such as *Bacteroides*, *Escherichia*, *Eubacterium*, *Lactobacillus*, and *Ruminococcus*, compared to healthy controls (Ott et al., 2008). In addition, Machiels et al. (2014) evidenced lower counts of *F. prausnitzii*, also observed in CD patients, and *Roseburia hominis*, both well-known butyrate-producing bacteria of the Firmicutes phylum, in UC subjects compared to healthy controls.

Patients with irritable bowel syndrome (IBS) revealed an altered microbial composition characterized by a higher abundance in Proteobacteria and Firmicutes and a lower abundance in Actinobacteria and Bacteroidetes compared to healthy controls; within Firmucutes, Lachnospiracae family and *Lactobacillus* genus were found more abundant in IBS patients (Carroll et al., 2010; Krogius-Kurikka et al., 2009).

Coeliac disease is a chronic gastrointestinal tract disorder showing damages at the small intestine, which are hypothetically linked to an autoimmune response caused by gluten ingestion in genetic predisposed subjects (Reilly and Fasano, 2012). Some authors evidenced an intestinal dysbiosis in coeliac patients with active disease characterized by a remarkable reduction in Gram positive bacterial population faecal specimens facilitating the colonization of potentially harmful Gram negative bacteria within the mucosal surface (Collado et al., 2009; Di Cagno et al., 2011). In particular, data obtained from duodenal biopsies revealed a reduction in the number of bifidobacteria and changes in species distribution by Denaturing Gradient Gel Electrophoresis (DGGE) within the *Bifidobacterium* genus (Nadal et al., 2007; Carmen and Hernández, 2007). In addition, coeliac subjects showed significantly reduced counts of members belonging to

Clostridium genus and *F. prausnitzii* in comparison with healthy controls; levels of IgA coating the *Bacteroides-Prevotella* group were found significantly reduced in coeliac subjects, evidencing the effects of the disease on the immunitary response-microbiota interaction (De Palma et al., 2010).
Patients with colon rectal cancer, which is the third most commonly diagnosed cancer among both men and women in the United States (Siegel et al., 2017), showed significantly higher abundance of Proteobacteria and lower abundance of Bacteroidetes compared to controls; at the genus level, increased abundance of *Dorea* and *Faecalibacterium* and lower proportions of *Bacteroides* and *Coprococcus* have been detected in patients compared to controls (Shen et al., 2010).
Imbalance in the gut microbiota composition has been suggested to play a role also in the pathogenesis of infant colic, a common disorder in the first 3 months of childhood. Colicky infants have a reduced faecal-bacterial diversity and stability, compared to the healthy ones; they also show a higher prevalence of Gram negative bacteria, especially coliforms, and a reduced abundance of beneficial bacteria, such as lactobacilli and bifidobacteria (Dubois and Gregory, 2016).
Among infant diseases, necrotizing enterocolitis (NEC) is probably the most devastating one and affects the intestine of mostly premature infants. Although the exact cause is unknown, the critical elements are thought to be an immature gastrointestinal tract mucosal barrier, an inappropriate pro-inflammatory response and altered bacterial colonization (Claud and Walker, 2001). Patients with NEC show an increase in abundance of gammaproteobacteria and a decrease in other bacteria species, especially bifidobacteria (Wang et al., 2009; Arboleya et al., 2012).

2.2 Dysbiosis in metabolic disorders

The gut microbiota, considering its role in host metabolism and its ability to harvest energy from food, is a significant factor in the risk of metabolic diseases.
A dysbiotic condition associated to a hyperlipidic diet can promote a chronic inflammatory response contributing to the pathogenesis of obesity (Sun et al., 2018). GF mouse models have provided invaluable insights into the link between gut microbiota and body fat accumulation in the host. GF mice develop less body fat than conventional mice, despite increasing their food intake and decreasing their metabolic rate (Backhed et al., 2007). Furthermore, GF mice colonised with an 'obese-microbiota' isolated from genetically obese ob/ob mice revealed a greater percentage increase in body fat than those colonized with a 'lean microbiota' (Turnbaugh et al., 2006).
Some human studies have shown the interaction between gut microbiota and the increase of body fat. Le Chatelier et al. (2013) evidenced that obese subjects showing low bacterial richness were characterised by a high adipose profile, high insulin resistance, dyslipidemia and high rates of systemic inflammation. The significant differences in bacterial richness between obese and lean groups in this study were based on 46 genera. Among them, *Bacteroides*, *Parabacteroides*, *Ruminococcus*, *Campylobacter*, *Dialister*, *Porphyromonas*, *Staphylococcus* and *Anaerostipes* were more dominant in obese subjects, while *Faecalibacterium*, *Bifidobacterium*, *Lactobacillus*, *Butyrivibrio*, *Alistipes*, *Akkermansia*, *Coprococcus* and *Methanobrevibacter* were more prevalent in lean subjects. Consequently, also functions associated to the gut microbiota result compromised in obese subjects. In fact, the same study revealed a reduction in butyrate-producing bacteria, a reduction in hydrogen and methane production, and increase in mucus degradation in obese subjects. An interesting prospective follow-up study by Kalliomaki et al. (2008) highlighted that alterations of the intestinal microbiota in the early stage of life were also associated to a higher risk

to develop overweight or obesity during life. In particular, bifidobacterial counts in faecal samples from infants were higher in children remaining normal weight than in children becoming overweight, whereas *Staphylococcus aureus* counts were higher in children becoming overweight than in children remaining normal weight.

Besides genetic predisposition and environmental factors, alteration in gut microbiota composition seemed to have a certain impact on type 1 diabetes onset. High level of Bacteroidetes with respect to Firmicutes were found in children, who developed this pathology later in life (Giongo et al., 2011). Furthermore, butyrate producers, as *Faecalibacterium* and *Roseburia*, and mucin degraders, as *Prevotella* and *Akkermansia,* were found in greater proportions in healthy controls compared with type 1 diabetic patients and have been described as protective against this disease (Brown et al., 2011; Murri et al., 2013).

Increased body weight is recognized as the greatest risk factor of type 2 diabetes, aside from genetic and lifestyle factors. It has been demonstrated that patients with this pathology have an altered gut microbial profile, marked by decreased butyrate-producing bacteria such as *R. intestinalis* and *F. prausnitzii* (Qin et al., 2012). Moreover, the gut microbial profile of type 2 diabetic patients is characterized by the presence of opportunistic pathogens such as *Bacteroides caccae,* various Clostridiales, *E. coli* and *Desulfovibrio* (Qin et al., 2012).

2.3 Dysbiosis in Central Nervous System (CNS)-related disorders

The gut microbiota is able to affect the CNS through a complex bidirectional communication system, which constitutes the gut-brain axis and involves different mechanisms, such as the alteration in circulating levels of inflammatory cytokines or the production of neurotoxins (Rhee et al., 2009). Dysbiosis may impact on the gut-brain axis leading to dysfunction in this interaction that may be implicated in the development and prognosis of neurological diseases. It is known that enteric infections can cause anxiety, depression, and cognitive dysfunction; GF mice display alterations in stress-responsively, central neurochemistry, and behaviours that can be interpreted as anxiolytic (Neufeld et al., 2011).

Although the pathogenesis of autism remains still obscure, it has been demonstrated that a higher number of species belonging to the *Clostridium* genus (10 times more) characterize the qualitative composition of fecal samples of autistic children (Song et al., 2004; Parracho et al., 2005). A more recent study carried out by Strati et al. (2017) demonstrated that constipated autistic individuals are characterized by high levels of bacterial taxa belonging to *Escherichia/Shigella* and *Clostridium cluster XVIII*. The same authors concluded that also the mycobiota may contribute in the developing of this disease, as the relative abundance of the fungal genus *Candida* has been found more than double in autistic subjects with respect to healthy controls (Strati et al., 2017).

A study that examined the gut microbiota in the faeces of Parkinson's disease (PD) patients showed a low abundance of Prevotellaceae, and a positive correlation between the level of Enterobacteriaceae and the worst motor phenotype (Scheperjans et al., 2015).

A recent study in China observed that the abundance of Lachnospiraceae was reduced by 42.9% in patients with PD, whereas the abundance of Bifidobacteriaceae was enriched in patients with PD, not identifying a significant difference in the overall microbial composition among different PD motor phenotypes, but an association between specific taxa and different PD motor phenotypes (Lin et al., 2018).

A recent study regarding microbiota in patients with Alzheimer's diseases (AD) showed that the composition of gut microbiota was different between patients and healthy controls. Several bacteria taxa in AD patients were different from those in controls at taxonomic levels, such as Bacteroides, Actinobacteria, Ruminococcus, Lachnospiraceae, and Selenomonadales, suggesting that gut microbiota is altered in AD patients and may be involved in the pathogenesis of AD (Zhuang et al, 2018).

Emerging avant-garde pre-clinical and clinical studies are focusing on the relationship between gut microbiota and amyotrophic lateral sclerosis (ALS). Brenner et al. (2018) observed significant differences between patients and controls in in the number of microbial species and in the abundance of uncultured Ruminococcaceae. Rowin et al. (2017) found besides signs of intestinal inflammation, a lower microbial diversity in the gut microbiota of diseased subjects compared with controls, in particular lower *Ruminococcus* spp. levels.

3. PROBIOTICS

3.1 Probiotics as "therapeutic microbiology"

The term "probiotic" means "for life" and it is currently used to name bacteria associated with beneficial effects for humans and animals. In 2001 the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) defined them as "live microorganism which, when administered in adequate amounts confer a health benefit on the host". This definition has been revised in 2014 by the International Scientific Association for Probiotics and Prebiotics, including in the term probiotic "microorganism for which there are scientific evidence of safety and efficacy" and excluding "live cultures associated with fermented foods for which there is no evidence of a health benefit" (Hill et al., 2014). The first intuition of the healthy potential of some selected bacteria is attributed to Eli Metchnikoff, the Russian Nobel Prize working at the Pasteur Institute at the beginning of the last century; in his book "*The prolongation of life*" (1908), he highlighted the health benefits of lactic acid bacteria related to ferment milk products. The concept of using microorganisms to treat or prevent targeted diseases has rapidly evolved inspiring a new branch of applied microbiology known as "therapeutic microbiology" (Spinler and Versalovic, 2008). Since the host and the gut microbiota establish an intimate symbiotic relationship that is fundamental for the maintenance of the host's health, the administration of beneficial microorganisms may represent a key determinant of the general health status and diseases susceptibility.

Probiotics that have been largely studied in humans mainly include species of the *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Saccharomyces* genera. Probiotics exert their action on the intestinal environment, affecting the entire health status of the host, with different mechanisms, which have been largely exposed by Fuller (1991):

- antagonism against pathogens with the production of antibacterial molecules, competition for nutrients, especially carbohydrates and polysaccharides, competition for binding to specific receptors on intestinal epithelium;
- alteration of microbial metabolism by increasing the activity of useful enzymes and decreasing the activity of some enzymes that can be dangerous for the host;
- stimulation of the immune system with different mechanisms, such as by increasing antibody and anti-inflammatory cytokines levels, and inducing macrophages and natural killer cells (MacDonald and Monteleone, 2005).

A probiotic formulation can contain just one strain, a mixture of different strains of the same species or it can be a multi-strain and multi-species mixture that, acting with a synergic effect, may enhance the effectiveness of each single strain (Chapman et al., 2011; Timmerman et al., 2004).

3.2 Probotics in gastrointestinal tract-related diseases

Several investigations have focused on the application of probiotics in these disorders. Many studies have shown the beneficial effects of *Lactobacillus reuteri*, one of the most used probiotics in infants, for the prevention and treatment of infant gastrointestinal disorders, including colics, regurgitation, vomit, constipation (Indrio et al., 2014; Chau et al., 2015). This species has been demonstrated to improve symptoms and reduce the number of anaerobic Gram negative bacteria, Enterobacteriaceae and enterococci in colicky infants (Savino et al., 2010; Savino et al., 2015).

Furthermore, *L. reuteri* ATCC 55730 has been found to be effective in children with distal active UC improving mucosal inflammation and modulating mucosal expression levels of some cytokines involved in the bowel inflammation (Oliva et al., 2012). Positive effects were also associated to *Lactobacillus* and *Saccharomyces* strains administered within a rehydration therapy for infectious diarrhoea in children (Allen et al., 2011). *Bifidobacterium* strains proved their efficacy against acute rotavirus diarrhoea in hospitalized children (Grandy et al., 2010; Vandenplas et al., 2011) and in preterm and low birth weight infants, highlighting clinical positive effects for the treatment of NEC (Khailova et al., 2009; Underwood et al., 2011). Particularly, *Bifidobacterium breve* YIT4010 has been proven to be effective in reducing abdominal symptoms, improving weight gain and establishing a correct microbial colonization in order to prevent infection in preterm infants (Kitajima et al., 1997; Li et al., 2004).

The contribution of probiotics in inducing or prolonging a remission condition in patients with IBD has been demonstrated. Scaldaferri et al. (2013) reviewed that *Lactobacillus* GG and *Saccharomyces boulardii* have been effective as adjuvant to standard therapy for the maintenance of remission in CD and UC. Several studies proved the efficacy of VSL#3, a probiotic formulation of four strains of *Lactobacillus* (*Lactobacillus paracasei*, *Lactobacillus plantarum*. *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*Bifidobacterium longum*, *Bifidobacterium infantis* and *B. breve*) and one strain of *Streptococcus salivarius* subsp. *thermophilus*, in UC patients. Bibiloni et al. (2005) showed that 6 weeks administration with this probiotic mixture improved UC remission and response in patients not responding to traditional therapy. VSL#3 efficacy was also tested in a study conducted on children with newly diagnosed UC (Miele et al., 2009). The same probiotic mixture has also been approved for the prevention and the maintenance of remission of pouchitis and the efficacy is stated also in referral European guidelines (Veerappan et al., 2012; Floch et al., 2011).

McFarland and Dublin (2008) reviewed 20 trials on probiotic treatments of IBS, revealing a general improvement of symptoms. Particularly, two different studies demonstrated the effectiveness of a mixture of *L. plantarum* and *B. breve* and VSL#3 in reducing IBS-associated pain (Saggioro, 2004; Kim et al., 2003).

As reviewed by Cristofori et al. (2018) despite the encouraging data deriving from *in vitro* studies, few *in vivo* data are available on probiotics supplementation in patients with coeliac disease. The administration of *B. infantis* revealed an improvement of gastrointestinal symptoms and an amelioration of the inflammatory state of coeliac subjects (Smecuol et al., 2013; Pinto-Sánchez et al., 2017). Olivares et al. (2014) showed that the administration of *B. longum* resulted in a reduction of Bacteriodes and Enterobacteriacea and an increase of harmless to potentially harmful bacteria ratio in gut microbiota of coeliac subjects, as well as an improvement of the inflammatory profile. Recently, a clinical trial conducted by Francavilla et al. (2018) evidenced an improvement of gastrointestinal symptoms together with a significant increase of bifidobacteria deriving from a treatment with a mixture bifidobacteria and lactobacilli strains administered in coeliac subjects with IBS symptoms.

3.3 Probotics in metabolic diseases

Current *in vitro* and *in vivo* studies demonstrated that probiotics can have anti-obesity and anti-inflammatory effects and the ability to improve glycaemic profile and modulate serum lipids (He and Shi, 2017). However, only a small number of studies focusing on human intervention were carried out to analyse the effects of a probiotic administration on body fat and weight. Among these, Minami et al. (2015) demonstrated the positive role of *B. breve* B-3 administration resulting in a significant decrease of the fat mass and an amelioration of blood parameter in adults with a tendency for obesity. A treatment with *Lactobacillus gasseri* 2055 has been proven to reduce abdominal adiposity and body weight in subjects with high body mass index (BMI) and abdominal visceral fat area (Kadooka et al., 2010). VSL#3 administration in overweight subjects contributed to the improvement in lipid profile, insulin sensitivity and decrease in C-reactive protein (CRP), which is a general marker for inflammation and infection (Rajkumar et al., 2014). Two different studies evidenced a decrease of liver aminotransferase activity in patients with obesity related non-alcoholic fatty liver disease (NAFLD) administered with a mixture of *L. bulgaricus* and *Streptococcus thermophilus* and with *L. rhamnosus* respectively (Loguercio et al., 2005; Vajro et al., 2011). The administration of *B. longum* for the treatment of non-alcoholic steatohepatitis (NASH) led to a significant improvement of liver histology pattern (Malaguarnera et al., 2012).
Asemi et al. (2013) evidenced that multispecies probiotic supplementation, including *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus*, in type 2 diabetic patients prevented a rise in fasting plasma glucose and resulted in a decrease of CRP and an increase in plasma total glutathione, which is note to have anti-oxidant effects. Ejtahed et al. (2012) showed that probiotic yogurt containing *L. acidophilus* La5 and *Bifidobacterium lactis* Bb12 influenced glycaemic levels reducing fasting blood glucose and glycated haemoglobin and improved the antioxidant status in type 2 diabetic patients. An improvement of the inflammatory status has been detected in patients with type 2 diabetes and NAFLD supplemented with a multi-strain probiotic mixture comprising strains belonging to *Bifidobacterium*, *Lactobacillus*, *Lactococcus* and *Propionibacterium* genera as coadjuvant of the standard antidiabetic therapy (Mykhal'chyshyn et al., 2016).
Several studies reviewed by Kobyliak et al. (2016) reported that dietary intervention with yogurt containing probiotics (*Enterococcus faecium*, *S. thermophiles*, *L. acidophilus*, *B. longum*, *L. plantarum* and/or *B. lactis*) significantly reduce total serum cholesterol and LDL-cholesterol and improve the LDL:HDL cholesterol ratio. A significant reduction in total and LDL-cholesterol has also been attributed to the intake of *L. gasseri* and inulin among hypercholesterolemic patients (He and Shi, 2017).

3.4 Probiotics in neurological diseases

As mood and behaviour disorders may be caused by a chemical imbalance in the brain, a probiotic administration can improve the production and the delivery of neuroactive compounds acting on the brain-gut axis and restoring the stability on nervous system. Many investigations assessed the potential of probiotics in preventing symptoms or alleviating comorbidities against neurological diseases, although the possible role of probiotics in some other neurological disorders, whose onset is obscure, as ALS, has to be still evaluated and a pioneer study in this sense has been conducted within my PhD work.

The administration of *L. casei* Shirota in chronic fatigue patients resulted in increase of lactobacilli and bifidobacteria in the gut microbial population and in decrease of anxiety symptoms (Rao et al., 2009). In addition, *L. brevis* SBC8803 supplementation in males with poor quality of sleep was found to restore the sleep tone (Nakakita et al., 2016).
Probiotics showed positive effects in neuroinflammatory and autoimmune disorders. The treatment with a probiotic mixture consisting of *L. acidophilus*, *L. casei*, *Bifidobacterium bifidum*, *L. fermentum* were shown to improve cognitive functions in AD patients (Akbari et al., 2016). The same formulation was effective also in multiple sclerosis patients in improving mental health condition (Kouchaki et al., 2017).
Probiotics can also assume the role of "psychobiotics". The first definition of psychobiotic was given by Dinan et al (2013): "a live microorganism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric hilliness". A probiotic formulation containing *L. acidophilus*, *L. casei* and *B. bifidum* administered in patients with major depressive disorders has been shown a general improvement of clinical picture (Akkasheh et al., 2016). Moreover, the supplementation of *L. rhamnosus* HN001 has been demonstrated to be effective in lowering *post-partum* depression and anxiety scores in women supplemented from 14-16 weeks gestation to 6 months *post-partum* (Slykerman et al., 2017).

3.5 Probiotics in other diseases

As already outlined in the previous paragraph, probiotics can show their beneficial effects in inflammatory diseases or conditions, such as post-surgery, apparently not related to the gastro-intestinal tract.
An improvement of the inflammatory status was observed in psoriasis patients receiving *B. infantis* 35624 supplementation, in children with mild and moderate atopic dermatitis receiving *L. plantatum* IS-10506, in adults with atopic dermatitis receiving *L. acidophilus* L-92, as reviewed by Kiousi et al. (2019). A formulation containing *B. breve* M-19V and *B. longum* BB536 administered to both mothers and newborns has been demonstrated to be effective in reducing the risk of developing eczema and atopic dermatitis in infants (Enomoto et al., 2014).
The administration of *L. casei* Shirota in knee osteoarthritis patients led to a reduction of the inflammatory status (Lei et al., 2017).
Supplementation with *B. breve*, as single strain or in association with other species, to children subjected to surgical procedure, has been observed to reduce the incidence of infection preventing or correcting abnormal microbial colonisations in gut microbiota (Mizuno et al., 2010; Kanamori et al., 2001; Kanamori et al., 2010).
Moreover, the administration of *B. breve* Yakult was found to be effective in reducing febrile episodes in cancer paediatric subjects (Wada et al., 2010).

OBJECTIVES

My PhD work was supported by the EU project FOODstars (Innovative Food Product Development Cycle: Frame for Stepping Up Research Excellence of FINS, GA 692276). The goal of FOODstars was to develop ideas for new, safe and innovative products for the food and nutraceutical industry that can improve the health status of healthy and diseased patients.
The gut microbiota, as already elucidated in the previous part of this thesis, establishes a symbiotic relationship with the host since birth contributing to the maintenance of the host's health status. It is well known that alterations of the normal microbial composition are implicated in the pathogenesis of some diseases; however, there are some disorders, whose aetiology is still obscure, in which the role of gut microbiota has not been fully clarified. As the gut microbiota is involved in several health-promoting functions, manipulation of its composition can have a positive influence on disease progression and patient health status. However, the effectiveness of some probiotic formulations, although already assessed in *in vitro* and/or in animal studies, needs to be validated in humans.

Therefore, the main aim of the work proposed in this thesis was to explore the possibility of probiotic supplementation for the prevention/treatment of diseases of different origin in humans.
The specific objectives of the work are:
- to study the gut microbiota of coeliac paediatric subjects and evaluate the effect of a *B. breve* supplementation on the disease progression;
- to evaluate the effect of *B. breve* supplementation for the prevention of functional intestinal disorders in newborns;
- to evaluate the effect of *B. breve* supplementation in the progression of obesity in children;
- to study the gut microbiota of ALS patients and evaluate the effect of lactic acid bacteria supplementation on the disease progression;
- to study the gut bacteria and bacteriophage population of preterm infants and how their composition is influenced by antibiotic treatments.

A brief outline of the rationale and activities performed within this work are described and schematized below.

✓ Several *Bifidobacterium* species are largely used as probiotics for their capability of reaching and colonizing the gastrointestinal tract and their documented history of safety. Among them, the species *Bifidobacterium breve,* originally isolated from infant faeces, represents one of the most used probiotics in infants and children. The first step of this work was to review the applications of *B. breve* strains mainly for the prevention/treatment of paediatric pathologies, starting from *in vitro* and mice model assessment of efficacy to the clinical use (**Paper 1**)

✓ Several studies, as reviewed in Paper 1, demonstrated the efficacy of *B. breve,* for the treatment of different infant diseases. Particularly, *in vitro* studies evidenced the potential of 2 strains of *B. breve,* B632 and BR03, as probiotics. These two strains revealed also to be effective in colonizing the gut of healthy newborns and in decreasing total faecal coliforms. The following step was to evaluate if this formulation was also effective in preventing or treating paediatric diseases

(**Paper 2**, **3**, **4**, **5**). *B. breve* B632 and BR03 are now commercialized as a probiotic formulation, named Bifibaby ®, by Probiotical S.p.A., Novara.

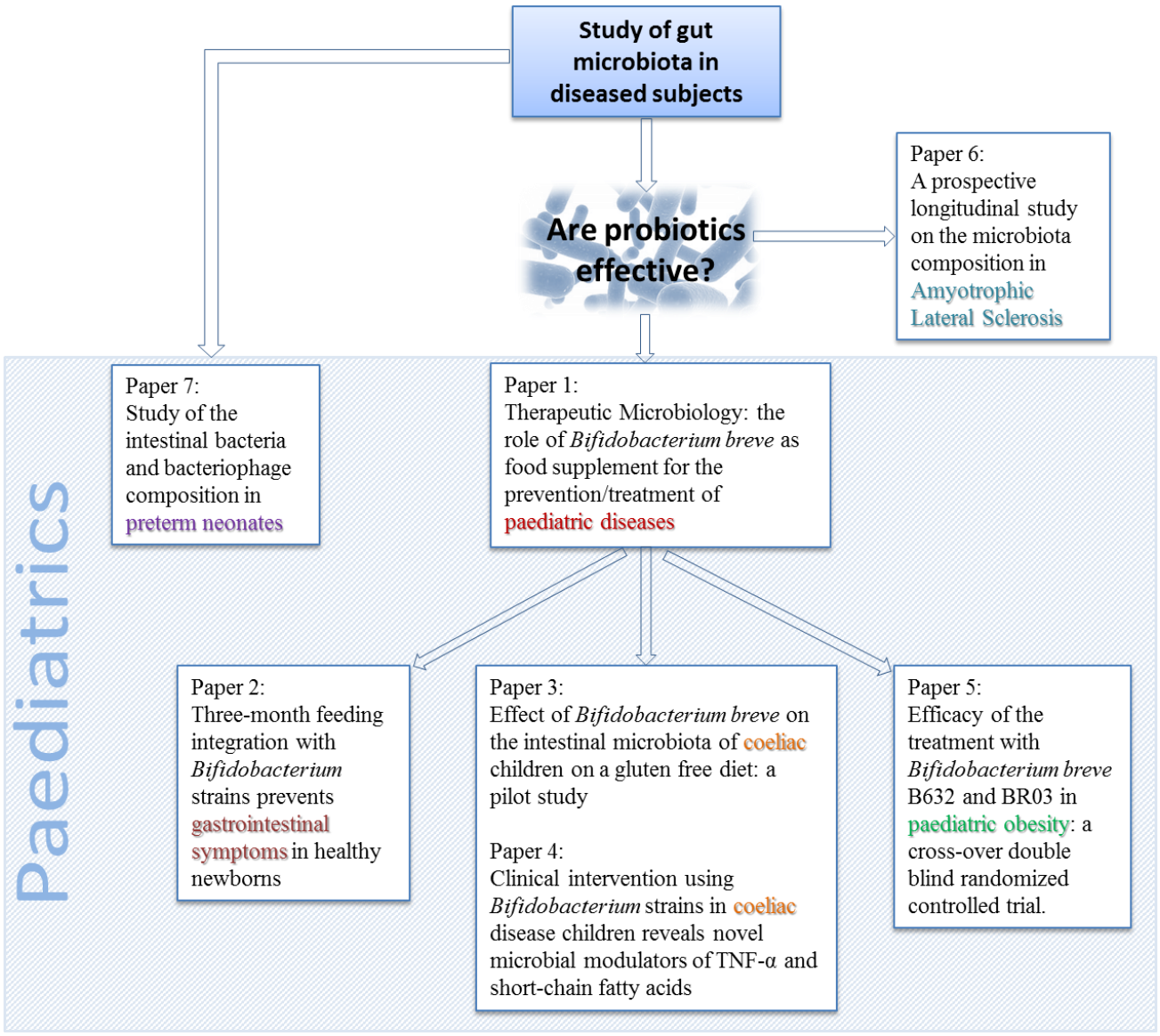
Infantile functional gastrointestinal disorders are common in the first months of life. Although the aetiology of these disorders has not been fully clarified, the gut microbiota and the type of feeding have been suggested to play a role in the pathogenesis of these conditions. **Paper 2** evaluated the effects of *B. breve* B632 and BR03 administered both to breast-fed and bottle-fed newborns in shifting the counts of targeted faecal microbial groups and preventing functional gastrointestinal disorders in a cohort of healthy newborns.

Coeliac disease has also been associated to an altered composition of the intestinal microbiota, evidencing a remarkable reduction in Gram positive bacterial population in duodenal and faecal specimens and facilitating the colonization of potentially harmful Gram negative bacteria within the mucosal surface. Until now only few studies have been focused on the administration of bifidobacteria in coeliac subjects. **Paper 3** and **4** evaluated the impact of the administration of *B. breve* B632 and BR03 on the gut microbiota composition, the inflammatory status, in particular TNFα levels, and faecal SCFAs in coeliac paediatric subjects compliant to a gluten-free diet. At the same time, these parameters were also compared to those of healthy subjects.

Obesity is a chronic and multifactorial condition and it is associated with a high risk of developing chronic comorbidities, especially in childhood. The involvement of gut microbiota in the excess of adiposity and the increase of body fat has been largely documented in studies carried out on mice models and humans. However, the impact of a manipulation of the gut microbiota through probiotics in obesity and its associated comorbidities has been scarcely investigated. **Paper 5** assessed the efficacy of dietary supplementation with *B. breve* B632 and BR03 on the clinical and metabolic profile and on the main gut microbiota groups in obese children and adolescents on a diet therapy.

✓ The imbalance of the intestinal microbial environment can have an impact also in disorders not directly related to the gastrointestinal tract, such as neurological diseases, affecting the gut-brain axis. The circulating levels of inflammatory cytokines and the production of neurotoxins, which can be compromised in a dysbiosis condition, constitute some of the mechanisms involved in ALS pathogenesis. **Paper 6** is the first prospective longitudinal study addressing the microbiota composition in ALS patients and matched healthy controls and considering the impact of a probiotic supplementation on the gut microbiota and disease progression.

✓ The last part of my work was dedicated to a preliminary study of gut microbiota of preterm infants, subjected to perinatal antibiotic treatments of different duration, finalized at the development of targeted probiotic treatments (**Paper 7**). Particularly, the whole bacterial composition of the intestinal tract as well as the related bacteriophage community were assessed in order to reach a global knowledge of the gut microbiome and phageome that characterize prematurity.


Study of gut microbiota in diseased subjects
Are probiotics effective?
Paper 6:
A prospective longitudinal study on the microbiota composition in Amyotrophic Lateral Sclerosis
Paediatrics
Paper 7:
Study of the intestinal bacteria and bacteriophage composition in preterm neonates
Paper 1:
Therapeutic Microbiology: the role of *Bifidobacterium breve* as food supplement for the prevention/treatment of paediatric diseases
Paper 2:
Three-month feeding integration with *Bifidobacterium* strains prevents gastrointestinal symptoms in healthy newborns
Paper 3:
Effect of *Bifidobacterium breve* on the intestinal microbiota of coeliac children on a gluten free diet: a pilot study
Paper 4:
Clinical intervention using *Bifidobacterium* strains in coeliac disease children reveals novel microbial modulators of TNF-α and short-chain fatty acids
Paper 5:
Efficacy of the treatment with *Bifidobacterium breve* B632 and BR03 in paediatric obesity: a cross-over double blind randomized controlled trial.

Paper 1

Therapeutic Microbiology: the role of *Bifidobacterium breve* as food supplement for the prevention/treatment of paediatric diseases

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Abstract: The human intestinal microbiota, establishing a symbiotic relationship with the host, plays a significant role for human health. It is also well known that a disease status is frequently characterized by a dysbiotic condition of the gut microbiota. A probiotic treatment can represent an alternative therapy for enteric disorders and human pathologies not apparently linked to the gastrointestinal tract. Among bifidobacteria, strains of the species *Bifidobacterium breve* are widely used in paediatrics. *B. breve* is the dominant species in the gut of breast-fed infants and it has also been isolated from human milk. It has antimicrobial activity against human pathogens, it does not possess transmissible antibiotic resistance traits, it is not cytotoxic and it has immuno-stimulating abilities. This review describes the applications of *B. breve* strains mainly for the prevention/treatment of paediatric pathologies. The target pathologies range from widespread gut diseases, including diarrhoea and infant colic, to coeliac disease, obesity, allergic and neurological disorders. Moreover, *B. breve* strains are used for the prevention of side infections in preterm newborns and during antibiotic treatments or chemotherapy. With this documentation, we hope to increase knowledge on this species to boost the interest in the emerging discipline known as "therapeutic microbiology."

Keywords: *Bifidobacterium breve*; probiotics; paediatrics; therapeutic microbiology

1. Introduction

The use of microorganisms to treat or prevent targeted diseases was conceived at the end of the last millennium. This concept has rapidly evolved giving rise to a new branch of applied microbiology known as "therapeutic microbiology" [1]. Since human organisms and gut microbiota establish an intimate symbiotic relationship that is fundamental for the maintenance of the host's health, the administration of beneficial microorganisms may represent a key determinant of the general health status and diseases susceptibility. The choice for the most suitable species for a certain pathology requires extensive studies, both *in vitro* and *in vivo*. Moreover, it is known that strains belonging to the same species may express different functions *in vivo* [2]. It has also been demonstrated that blending different microbial strains, species or even genera, may lead to a final effect that is not predicted by results from using each single microorganism. Several *Bifidobacterium* species are largely used as probiotics for their capability of reaching and colonizing the gastrointestinal tract and their documented history of safety. Among them, *Bifidobacterium breve*, originally isolated from infant faeces, represents one of the most used probiotics in infants. The multiple studies in which *B. breve* strains have been successfully used in diseased humans, especially children and newborns, witness the potentiality of strains belonging to this species for the prevention or treatment of human diseases. The aim of this review is to show the various applications of *B. breve* for preventing and treating paediatric diseases starting from in vitro and mice model assessment of efficacy to the clinical use. To the best of our knowledge, this work represents the first collection of works focused on the application in paediatrics of strains belonging to the *B. breve* species and is aimed to shed light on the role of this *Bifidobacterium* species in the scenario of "therapeutic microbiology." Moreover, this paper explores the effectiveness of *B. breve* used both as a single strain and combined with other microorganisms with a final short outcome of its application in adulthood.

2. The Human Intestinal Microbiota

The human intestinal microbiota is a complex ecosystem that includes not only bacteria but also fungi, Archaea, viruses and protozoans; bacteria concentration increases from the stomach and duodenum throughout the intestinal tract and in the large intestine it rises to 10^{11}–10^{12} CFU/g of lumen content [3]. It has been estimated that at least 1800 genera and a range of 15,000–36,000 bacterial species, depending on whether species are conservatively (97% OTUs) or liberally (99% OTUs) classified, can be found in the large intestine [4].

The symbiotic mutualistic relationship that the gut microbiota establishes with the host exerts several beneficial roles, the main of which are the maintenance of the gut epithelial barrier, the inhibition of pathogen adhesion to intestinal surfaces, the modulation and proper maturation of the immune system, the degradation of otherwise non-digestible carbon sources such as plant polysaccharides and the production of different metabolites including vitamins and short chain fatty acids (SCFAs) [5]. Furthermore, intestinal microorganisms seem to be responsible for a bidirectional interaction between the gut and the Central Nervous System (CNS) via the gut-brain axis [6]. Dysfunction in this interaction may be implicated in the development and prognosis of some neurological diseases, including autism [7], multiple sclerosis [8] or Parkinson disease [9]. Because of this symbiotic relationship, the human organism can be seen as a "superorganism," which consist of not only the microbial cells but also their genomes, that is, the microbiome and the related microproteome and micrometabolome [10]. The microbiome represents more than 100 times

the human genome (1,000,000 genes vs. 23,000 genes) [10]. Indeed, the gut microbiome is influenced by external factors, such as diet, health status and xeno-metabolome. These factors shape the individual intestinal microbiota that can be considered as a "fingerprint" of the hosting organism.

Recently, the realization of global-collaborative projects has enriched the knowledge about the gut microbiota, such as the MetaHit project [11], the Human Microbiome project [12] and the MyNewGut project [13]. Moreover, the large amount of data from high throughput gene sequencing technology has allowed us to gain deeper insights in the composition of the "typical" human gut microbiota. The two principal bacterial phyla are *Firmicutes* and *Bacteroidetes*, followed by *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. Fungi and Archaea constitute approximately 1% of the species of the intestinal microbiota [14,15]. The predominant fungal phyla are *Ascomycota* and *Basydiomicota*; some of the most abundant genera, that is, *Saccharomyces*, *Debaryomyces* and *Kluyveromyces*, are found in food, confirming the influence of diet habits also on the fungal intestinal population [16]. From the 80s some archaeal species belonging to *Methanobrevibacter* genus have been identified. *Methanobrevibacter* is the only genus detected in the gut probably due to the use of 16S primers not having sufficient resolution for Archaea. Within this genus, the species' composition depends on diet and host's health status, as for the entire microbiota [17,18].

Microbiologists' attention has been also focused on bacteriophages, which, living at bacteria expense and being vehicles of genetic transfer, could have an important role in shaping the biodiversity of the gut ecosystem. The first metagenomic analysis of an uncultured viral community from human faeces using partial shotgun sequencing suggested a large diversity of phages in gut microbiota [19]. The same authors investigated the viral community in the infant intestine using metagenomic sequencing: 72% of the detected viral community resulted to be siphoviruses and prophages and over 25% resulted to be phages that infect lactic acid bacteria; faecal viral sequences were not identified in breast milk, suggesting a non-dietary initial source of viruses [20]. The entire viral community composition changed dramatically between the first and the second week of age [20], remaining then stable during host's life [21].

Gut colonization begins at birth, although recent evidences suggest the existence of an intrauterine transmission of maternal bacteria to the foetus [22]. The first colonizer are facultative anaerobes (*Staphylococcus* spp., *Enterobacteriaceae* and *Streptococcus* spp.), followed by strict anaerobes, such as members of *Bifidobacterium*, *Bacteroides* and *Clostridium* genera [23,24]. The mode of delivery exerts a strong influence on the first microbial colonization of newborns' gut. Children born by natural delivery have an intestinal microbiota profile similar to their mother's vaginal one, characterized by *Lactobacillus* and *Prevotella* spp., while children born by caesarean section develop a microbiota similar to that of mother's skin (*Streptococcus*, *Corynebacterium* and *Propionibacterium* spp.) [25]. In addition, the type of feeding has a crucial role on the colonization of microbial groups in the gut. Indeed, the gut microbiota of formula-fed infants contains a higher amount of *Escherichia*, *Veillonella*, *Enterococcus* and *Enterobacter* members and the concentration of *Lactobacillus* and *Bifidobacterium* is lower with respect to in breast-fed infants [26]. The abundance of these genera can be due to a more acidic pH in the colon of breast-fed infants [27]. The prevalence of bifidobacteria in breast-fed infants is also due to their capability of fermenting oligosaccharides (referred to as human milk oligosaccharides, HMO) [28]. Diet continues to exert a crucial influence in the gut microbiota composition also in adulthood: De Filippis et al. [29] showed

an association between plant-based diet and a prevalence of *Lachnospira* and *Prevotella* and a positive correlation between *Ruminococcus* and omnivore diet. Animal-based diets increase the abundance of bile-tolerant microorganism (*Alistipes*, *Bilophila* and *Bacteroides*) and decreases the levels of *Firmicutes* [30].

The use of antibiotics influences the gut microbiota composition, determining a significant decrease of the microbial diversity in the digestive tract [31,32]. However, the microbiota is a resilient system and tends to return to the pre-treatment state within 1 to 2 months after the end of the administration [33]. Moreover, the use of perinatal antibiotics, such as in the intrapartum prophylaxis, influences the establishment of a normal gut microbial composition and function, in particular reducing the levels of bifidobacteria and increasing potential pathogens [34–36].

It is well established that a functional and balanced microbiota reflects a healthy condition of the host; on the other hand, an unhealthy status may be associated with a compromised gut microbiota displaying a decrease of beneficial bacteria and increase of harmful ones.

3. Probiotics with a Special Emphasis on *Bifidobacterium breve*

"Probiotic" means "for life" and it is currently used to name bacteria associated with beneficial effects for humans and animals. In 2001 the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) defined them as "live microorganism which, when administered in adequate amounts confer a health benefit on the host" [37]. This definition has been revised in 2014 by the International Scientific Association for Probiotics and Prebiotics, including in the term probiotic "microorganism for which there are scientific evidence of safety and efficacy" and excluding "live cultures associated with fermented foods for which there is no evidence of a health benefit" [38].

Probiotics that have been largely studied in humans include species of the *Lactobacillus* and *Bifidobacterium* genera. Probiotic administration in the first stage of life results to be more effective in prevention and treatment of disorders, leading to a correct microbial colonization when the gut microbiota is still in a period of establishment. Several studies have shown the beneficial effects of *Lactobacillus reuteri*, one of the most used probiotics in infants, for the prevention and treatment of infant gastrointestinal disorders, including colics, regurgitation, vomit, constipation [39–41]. This species has been demonstrated to improve symptoms and reduce the number of anaerobic Gram negative bacteria, *Enterobacteriaceae* and enterococci in colicky infants [42,43]. Furthermore, *L. reuteri* ATCC 55730 was effective in children with distal active ulcerative colitis (UC) improving mucosal inflammation and modulating mucosal expression levels of some cytokines involved in the bowel inflammation [44]. *Lactobacillus* and *Saccharomyces* strains (*L. casei* CG, *L. reuteri* ATCC 55730 and a strain of *S. boulardii*) exerted positive effects as supplement for rehydration therapy for infectious diarrhoea in children by reducing the duration and stool frequency [45].

Several data are available for the use of bifidobacteria as probiotics for therapeutic purposes in infants [46]. As an example, *Bifidobacterium* strains belonging to the *animalis* (BB-12 strain) and *longum* species proved their efficacy against acute rotavirus diarrhoea in hospitalized children, particularly by increasing the immune response and decreasing duration of disease [47–49]. In addition, administration of *Bifidobacterium bifidum* and *B. animalis* strains in preterm and low birth weight infants demonstrated clinical positive effects for treatment of necrotizing enterocolitis (NEC) [50–52].

Among the different species belonging to the *Bifidobacterium* genus, *Bifidobacterium breve*, is the dominant one in breast-fed newborns [53] and one of the most used in infants. The species *B. breve* was firstly described by Reuter [54], who isolated from breast-fed infant faeces and named seven species of *Bifidobacterium*, including *B. parvulorum* and *B. breve*. The two species were then combined under the name of *B. breve* [55]. *B. breve* strains are also found in the vagina of healthy women [54]. Their presence in extra-body environments is a consequence of faecal contamination and the species is a useful indicator of human and animal faecal pollution [56]. *B. breve*, like other *Bifidobacterium* species, possess an array of enzymes for the utilization of different carbohydrates. These enzymes, useful to adapt and compete in an environment with changing nutritional conditions, are inducible in the presence of specific substrates. Amongst them, glycosidases, neuraminidases, glucosidases, galactosidase are included as well as extracellular glycosidases that degrade intestinal mucin oligosaccharides and glycosphingolipids [57]. *B. breve* also possess a glucosidase with a β-D-fucosidase activity, useful for the utilization of fucosilated HMO [58]. *B. breve* is included in the list of Qualified Presumption of Safety (QPS) biological agents [59]. Furthermore, recent studies have shown that human milk, traditionally considered as sterile, contains commensal, mutualistic and/or potentially probiotic bacteria for the infant gut. Among the different *Bifidobacterium* species found in human milk, *B. breve* strains have been detected with DNA-based techniques and also isolated and characterized [60]. These bacteria from human milk rapidly colonize the newborn's gut, protect the infant against infections and contribute to the maturation of the immune system [60].

Early studies by Akiyama et al. [61] showed that *B. breve* administration soon after birth was effective in developing a normal intestinal microbiota and, furthermore, *B. breve* showed a stronger affinity for immature bowel than other species, such as *B. longum*, evidencing its strong capabilities as probiotic. These achievements stimulated the development of further studies that gave new insights to the importance of this species as probiotic in infants.

Aloisio et al. [62] screened 46 *Bifidobacterium* strains for their capability of inhibiting the growth of gut pathogens including coliforms isolated from colicky infants. The most interesting strains belonged to the *B. breve* species, namely B632 strain (DSM 24706), B2274 strain (DSM 24707) and B7840 strain (DSM 24708). In addition to the antimicrobial activity against coliforms and other pathogenic bacteria, the strains did not possess transmissible antibiotic resistance traits and were not cytotoxic for gut epithelium, which are important pre-requisites for their use as probiotics. *B. breve* B632 was also able to stimulate the activity of mitochondrial dehydrogenases of macrophages and the production of IL-6, linked to a considerable activation of macrophages and endothelial cells in inflammatory condition. The potential of *B. breve* B632 as probiotic was also evidenced by Simone et al. [63]: it was able to inhibit the growth of *Enterobacteriaceae* in an in vitro gut model system stimulating the intestinal microbiota of a 2-month colicky infant, supporting the possibility to move to an in vivo study. Another strain of *B. breve*, BR03 (DSM 16604), revealed to be effective, as well as B632, in inhibiting the growth of 4 *E. coli* biotypes [64]. Mogna et al. [65] also underlined the validity of these two *B. breve* strains (B632 and BR03) in an *in vivo* study. The administration of both strains for 21 consecutive days as an oily suspension (daily dose of 100 million live cells of each strain) to healthy children was effective in obtaining gut colonization and in decreasing total faecal coliforms.

A biotechnological approach could improve the gastric transit survival, gastrointestinal persistence and therapeutic efficacy of the strain *B. breve* UCC2003, isolated from infant stool, via

the heterologous expression of the listerial betaine uptake system gene, BetL [66]. In addition to the improved capability of colonizing the intestine of inoculated mice, the strain was also able to reduce *Listeria* proliferation in the organs of the infected mice. Although the introduction of genes from pathogens into probiotic cultures is unlikely to meet approval from regulatory authorities, this study underlined that probiotic characteristics can be susceptible to improvements. Future perspectives include the obtainment of BetL homologues from Generally Recognized as Safe (GRAS) organisms and natural selection of probiotic cultures with elevated expression of such homologues.

B. breve strain Yakult (BBG-01) is another widely used probiotic strain. It was one of the first *B. breve* strain shown to possess the ability to modulate the intestinal microbiota by reducing the count of several pathogenic bacteria, such as *Campylobacter*, *Candida* and *Enterococcus* spp., after oral administration [67,68]. This strain has also displayed an anti-infective activity against Shiga-toxin-producing *E. coli* (STEC) O157:H7 in infected mice [69].

For its valid properties as probiotic, *B. breve* has also found a notable place in food technology in the fermentation of milk. In this regard, the positive effects associated to *B. breve*-fermented soymilk has been reported in several studies, demonstrating to improve lipid metabolism, alcohol metabolism and mammary carcinogenesis in mice models [70–72].

Moreover, a strain of *B. breve* has been included in a widespread of commercial high concentrated probiotic preparation, known as VSL#3, which contains 10^{11}–10^{12} viable lyophilized cells of different bacterial species that are usual component of human gut microbiota. Specifically, the formulation contains four strains of *Lactobacillus* (*L. paracasei*, *L. plantarum*. *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*B. longum*, *B. breve* and *B. infantis*) and one strain of *Streptococcus salivarius* subsp. *thermophilus*. VSL#3 exhibited an immunomodulatory capacity in in vitro studies by increasing the production of anti-inflammatory cytokines and inhibiting the production of pro-inflammatory cytokines [73].

4. *B. breve* Effectiveness in Mice Models

The strong evidence of the immune modulating capability of *B. breve* strains has been consolidated and well documented in a large number of animal models studies, which are the basis for human clinical trials.

The oral administration of *B. breve* YIT4064 strain, isolated from faeces of a healthy breast-fed infant, in mice immunized orally with an influenza virus was able to increase anti-influenza virus IgG levels in serum, thus protecting mice against infection. The authors concluded that the oral administration of this strain may enhance antigen-specific IgG against various pathogenic antigens taken orally and induce protection against various viral infections [74]. This conclusion was also supported by the study of Yasui et al. [75] that proved that the same strain stimulated anti-influenza virus hemagglutinin IgA by Peyer's patch cells in response to addition of hemagglutinins. These antibodies may reach the mucosal tissue and prevent influenza virus infection.

B. breve UCC2003 possessed a cell surface exopolysaccharide (EPS) able to play an important role in immunomodulation in B cell response. Administration for 3 consecutive days of EPS^+ *B. breve* strains in mice infected with *Citrobacter rodentium*, a diarrheagenic pathogen related to human *E. coli*, is effective in reducing the pathogen colonization, differently from mice fed with EPS^- *B. breve* [76]. EPS was involved in the production of a biofilm on the gut epithelium [77] preventing the attachment of *C. rodentium*.

Natividad et al. [78] illustrated the relationship between *B. breve* NCC2950 and regenerating (REG) III proteins, molecules belonging to the family of C-type lectins, which are expressed in the intestine and involved in maintaining gut homeostasis. The group REGIII-γ was measured in the ileum and colon of germ-free (GF) mice, mice colonized with specific pathogen free (SPF) microbiota and with a low diversity microbiota (altered Schaedler flora–ASF). Monocolonization with the probiotic *B. breve* NCC2950 but not with the commensal *E. coli* JM83, significantly induced REGIII-γ expression.

B. breve MRx0004, isolated from faeces of healthy humans, possessed a protective action in a severe asthma condition [79]. The study remarked an important decrease of neutrophil and eosinophil infiltration in lung bronchoalveolar lavage fluid in a mouse model of severe asthma after the probiotic treatment. This result, together with the demonstrated reduction of pro-inflammatory cytokines and chemokines involved in neutrophil migration, showed that *B. breve* MRx0004 effectiveness in reducing the above-mentioned inflammation condition paves the way for next-generation drug for management of severe asthma.

Many *B. breve* strains played an important role in prevention and treatment of various allergy conditions. Oral administration of *B. breve* M-16V, isolated from faecal sample of a healthy infant, in ovalbumin (OVA)-immunized mice significantly reduced the serum levels of total IgE, OVA-specific IgE and OVA-specific IgG1 and *ex vivo* production of IL-4 by the splenocytes [80]. Schouten et al. [81] showed that an intervention with a synbiotic formulation, comprising *B. breve* M-16V and a GOS/FOS mixture, was protective against the development of symptoms in mice orally sensitized with whey. The promising effect was confirmed by Kostadinova et al. [82] demonstrating the partially prevention of skin reaction due to cow's milk allergy, following the probiotic administration in combination with specific β-lactoglobulin—derived peptides and a specific blend of short- and long-chain fructo-oligosaccharides in mice. Particularly, the treatment, besides increasing the cecal content of propionic and butyric acid, determined an increase of IL-22 expression, which plays an antimicrobial role in the innate immunity response and of the anti-inflammatory cytokine IL-10 in the Peyer's patches. This outcome agrees with Jeon et al. [83], who demonstrated that the administration of the *B. breve* Yakult strain increased the number of IL-10-producing $CD4^+$ T cells in the large intestine of murine models and an increased production of acetic acid [69].

B. breve was also involved in protective mechanisms against obesity; the orally administration of *B. breve* B-3 in a mouse model with diet-induced obesity could suppress the increase of body weight and epididymal fat, with improved serum levels of total cholesterol, fasting glucose and insulin and act by regulating gene expression pathways involved in lipid metabolism and response to stress in the liver [84,85].

Increasing evidence suggests that a brain–gut–microbiome axis exists, although its role in cognition remains relatively unexplored [6,86]. Bifidobacteria were found to improve the behavioural deficits and to possess a potential action on stress-related disorders in model mice [87]. *B. breve* strains potential has also been investigated for the capability of conferring beneficial effects on neurological diseases. Savignac et al. [88] showed that 6 weeks feeding of *B. breve* 1205 strain resulted in positive effects on compulsive behaviour in marble burying test, anxiolytic effects in the elevated plus maze and reduced body weight gain in model mice, contributing to a general amelioration of anxiety and metabolism. Kobayashi et al. [89] showed that oral administration of *B. breve* A1, isolated from faeces of human infants, prevented cognitive decline in Alzheimer disease

(AD) model mice, with a reduction of neural inflammation; they observed that the probiotic provided ameliorations in both working memory and long-term memory. Furthermore, they found an increase of plasma acetate levels after the probiotic treatment and the neural inflammation reduction can be considered as a consequence of this increase due to *B. breve* administration, since SCFAs have been shown to have immune modulatory functions in model mice [90]. This evidence suggests that *B. breve* A1 has therapeutic potential for preventing cognitive impairment in Alzheimer disease and the necessity to move to a clinical intervention to evaluate the effects on diseased humans.

B. breve supplementation can affect the metabolism of fatty acids. Among them, eicosapentaenoic acid (EPA), which derives from α-linolenic acid metabolization, is an essential constituent of the cell membrane, plays an important role in brain and nervous system development and in inflammatory response [91]; docosahexanoic acid (DHA), which derives from EPA metabolization, is one of the major n-3 polyunsaturated fatty acids (PUFA) in the brain and is essential for a correct development of foetal encephalon [92]. Some studies revealed that human commensal microorganisms are able to synthetize bioactive isomers of conjugated linoleic acids (CLA) from free linoleic acid [93]; CLA was proven to possess antiatherosclerotic, antidiabetic and immunomodulatory properties [94,95]. Wall et al. [96] demonstrated that oral administration for 8 weeks to different animals (pigs and mice) of *B. breve* NCIMB 702258, a CLA producer strain, in combination with linoleic acid as substrate, increased the concentration of the predominant CLA isomer found in nature (*c*9, *t*11) in the liver. Furthermore, this supplementation in mice increased EPA and DHA levels in the adipose tissue and reduced proinflammatory cytokines tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) levels. The same authors demonstrated that a 8 weeks administration with the same *B. breve* strain and α-linolenic acid, the precursor of EPA, resulted in an increase in the liver EPA and brain DHA concentrations in mice. These results outline that the *B. breve* strain is a notable candidate for the treatment of inflammatory and neurodegenerative being able to modulate the hippocampal expression of brain-derived neutrophic factor (BDNF), a neurotrophin involved in development of the nervous system [97,98]. Particularly, the probiotic treatment reduced the expression of BDNF exon IV, which has been described as being highly responsive and increased by stress [99].

5. *B. breve* Application in Clinical Trials in Paediatrics

The use of *B. breve* strains for treatment and prevention of human diseases have been increasingly expanding in the last decade. Being bifidobacteria the most abundant bacterial group in infant gut, most of the studies are focused on paediatric subjects. Figure 1 summarizes the main applications of *B. breve* in paediatric diseases.

Therapeutic and protective role for human health of *B. breve* strains both as single strain or as a mixture of two strains of the same species has been demonstrated. As already mentioned, several researchers account for the improved efficacy of multi-species and multi-strain formulations that acting with a synergic effect, may enhance the effectiveness of each single strain [100,101].

Figure 1. Paediatric diseases in which an amelioration of symptoms has been obtained upon *B. breve* strains administration.

5.1. Preterm Infants and Necrotising Enterocolitis (NEC)

A consistent number of preterm infants, especially those of very low birth weight, are subjected to episodes of systemic infection caused by antibiotic resistant bacteria and fungi that can lead to chronic diseases and brain injuries [102,103]. These episodes can result from a combination of factors, including immature gastrointestinal tract mucosal barrier and undeveloped gastrointestinal tract immune system, which may predispose premature infants to bacterial translocation, causing systemic infection and necrotising enterocolitis (NEC) [104,105]. In addition, preterm infants have revealed an altered microbiota composition, resulting in almost undetectable bifidobacteria counts during the first and second week of life, differently for those at term [106–108]. This observation has allowed the formulation of the hypothesis that a bifidobacteria treatment could lead to a reintegration of beneficial bacteria in the intestinal environment and a reduction of bacterial translocation to other districts, stimulating researches in this sector. One of the first study that investigated the effects of a *B. breve* supplementation in preterm neonates reported that the strain YIT4010, administered as a suspension of distilled water containing 0.5×10^9 bacterial cells for 28 days, was able to colonize efficiently the intestinal tract, to reduce abnormal abdominal symptoms and to improve the weight gain [109]. A later study compared the effects of the administration of a *B. breve* strain a few hours after birth and 24 h after birth; the supplement was prepared by dissolving 1.6×10^8 cells in 0.5 mL of 5% glucose solution and administered twice a day for all the duration of hospitalization [110]. In newborns administered with the probiotic soon after birth, bifidobacteria were detected significantly earlier and the number of *Enterobacteriaceae* at 2 weeks after birth was significantly lower, compared to the infants treated 24 h after birth demonstrating that a very early probiotic intervention may contribute to the establishment of a beneficial gut microbiota and the prevention of infectious diseases [110].

A more recent work proved the suitability of *B. breve* M-16V administration for routine use in preterm infants in order to control the gut microbiota colonization and shift it towards a healthy profile [111]. Moreover, a retrospective cohort study was performed with the purpose of evaluating whether the supplementation with the same probiotic to preterm neonates would reduce the risk of NEC [112]. NEC represents the most life-threatening pathology of preterm neonates with incidence and mortality of 10–12% and 40–45%, respectively. It is characterized by gastrointestinal dysfunction progressing to pneumatosis intestinalis, systemic shock and rapid death in severe cases [113,114]. NEC is categorized into 3 different stages based on the severity of the disease, from stage *I*, a suspicion for disease, to stage *III*, corresponding to a severe progression of the disease [115]. Although the pathogenesis of this condition remains obscure, some important prevention strategies have been adopted, such as the use of antenatal glucocorticoids, early preferential feeding with breast-milk, prevention and treatment of infections [116]. Since preterm infants have shown an intestinal reduction of total bifidobacteria and a predominance of facultative anaerobes, some of which potentially pathogens, until the 20th day of life, it has been suggested that a major etiological factor for NEC could be an altered microbiota composition [117]. Therefore, a probiotic treatment can be an additional strategy for NEC prevention. A 3-week *B. breve* M-16V supplementation (3×10^9 CFU/day) has been associated with a lower incidence of NEC (≥stage *II*) in very low birth weight infants born before 34 weeks; the incidence in those born before 28 weeks resulted lower but not statistically significant [112]. Satoh et al. [118] had already demonstrated the efficacy of *B. breve* M-16V administration in preventing NEC in extremely low and very low birth weight infants: the probiotic was daily supplemented at a dose of 1×10^9 CFU dissolved in breast milk or breast-mixed with formula milk several hours after birth and continued until discharge from hospital (achievement of body weight 2300 g or gestational age of 37 weeks); the treatment led to a significant reduction of infection and mortality rate.

Various studies suggested that an overproduction of SCFAs in the intestinal environment can lead to mucosal injuries, which may evolve in NEC in premature infants [119,120]. Wang et al. [121] demonstrated that a 4 weeks *B. breve* M-16V supplementation (1.6×10^8 cells suspended in 0.5% glucose solution) was associated with a reduction of butyric acid levels in very and extremely low birth weight newborns. Since butyric acid increases the IL-8 secretion in enterocytes, condition that may lead to neutrophil invasion, a known hallmark of NEC, *B. breve* administration can be considered protective against NEC onset.

Immediately after delivery, some physiological changes, especially in the immunologic system, occur in newborns in order to adapt themselves to the new environment. *B. breve* M-16V, administered at 10^9 cells in 0.5 mL of 5% glucose solution starting several hours after birth, can increase the transforming growth factor β1 (TGF-β1) signals in preterm infants [122]. This increase has a relevant importance as it is known to induce oral tolerance, exert anti-inflammatory effects, express mucosal IgA and promote epithelial cell proliferation and differentiation [123]. A further study investigated the preventive effects of the same *B. breve* strain against infections and sepsis in extremely and very low birth weight newborns. The probiotic consisted on a freeze-dried preparation with a dose of 10^9 CFU dissolved in breast- or formula-milk; the development of infection and sepsis resulted significantly lower in the supplemented group compared with the non-supplemented one [124], highlighting once more the efficacy of a *B. breve* treatment in the prevention of developing infections, sepsis and NEC.

According to Braga et al. [125] the combined use of *B. breve* Yakult and *L. casei* was able to reduce the occurrence of NEC and was associated with an improvement in intestinal motility in newborns. The intervention started at the second day of life and continued for 30 days, provided *L. casei* and *B. breve* mixed to human milk in a daily dosage of 3.5×10^7 and 3.5×10^9 CFU, respectively. The number of NEC confirmed cases (≥stage *II*) was reduced upon probiotic treatment.

5.2. Gastrointestinal Disorders

A disorder that affects up to 30% of newborns in the first months of life is infant colic. It is characterized by paroxysmal, excessive and incontrollable crying without identifiable causes [126] representing a serious problem for the family and, in many cases, it can cause disorders later in life [127,128]. The aetiology remains obscure but an unbalanced intestinal microbiota has been suggested to play a role in the disease pathogenesis. Several studies support the use of probiotics as therapeutic or preventive agent against colics but very few clinical trials have been performed on bifidobacteria application. A mixture of *B. breve* strains (BR03 and B632), whose probiotic potential, as already highlighted in section 3, has been extensively demonstrated *in vitro*, was prepared as oily suspension and administered at a daily dosage of 5 drops containing 10^8 CFU of each strain to 83 infants, involving both breast and bottle-fed subjects [129]. Preliminary results showed that administration was effective in reducing minutes of daily crying. The clinical trial was then completed (155 infants, 130 breast- and 25 bottle-fed), as described in Aloisio et al. [130]; the *B. breve* mixture was able to prevent gastrointestinal disorders in healthy breast-fed infants, principally by reducing 56% of daily vomit frequency, decreasing 46.5% of daily evacuation over time and improving stool consistency. The strength of this study is the interrelation among a prolonged probiotic treatment, several clinical and anthropometric parameters (e.g., crying time, stool frequency, colour and consistency, regurgitation, vomits, weight, length, head circumference of newborn, delivery mode, type of feeding, gestational age) and main gut microbial groups. Epidemiological data have shown the predisposition of neonates born by caesarean section to develop obesity later in life [131,132]. However, the *B. breve* supplementation in infants born by caesarean section [130] resulted in a lower catch-up growth in weight, thus allowing the authors to speculate a protective effect of the probiotic strains against the risk to develop metabolic disturbance later in life.

Another common disease in childhood related to the intestinal tract is functional constipation, a chronic condition characterized by infrequent defecation (less than three times per week) and more than two episodes of faecal incontinence per week [133]; the pathogenesis, undoubtedly multifactorial, has not a well-defined aetiology. It has been shown that, despite intensive medical and behavioural therapy, 25% of patients developing constipation before the age of 5 years continue to have constipation upsets beyond puberty [134]. A pilot study showed the beneficial effects of 4 weeks treatment with *B. breve* Yakult (BBG-01) in constipated children: daily administration of 10^8–10^9 CFU led to a significantly increase in defecation frequency and amelioration of stool consistency, frequency of episodes of faecal incontinence and abdominal pain [135]. There is a debate of whether it is more effective the use of single strains or an association of them for constipation treatment; however, the mentioned study demonstrated that the intake of only one *B. breve* strain is even effective. Giannetti et al. [136] investigated the effects deriving from the administration of a mixture of 3 bifidobacteria, namely *B. infantis* M-63, *B. breve* M-16V and *B.*

longum BB536, in children suffering from irritable bowel syndrome (IBS). IBS is a functional bowel disorder characterized by chronic abdominal pain, discomfort, bloating and altered bowel habits including diarrhoea or constipation [137]. The daily dose was about 10^9 cells for each strain administered as bacterial powder and the treatment lasted 6 weeks. The bifidobacteria mixture intake resulted in a significant decrease in prevalence and frequency of abdominal pain and an improvement of the quality of life, assessed by an interview-administered validated questionnaire.

The commercial formulation VSL#3, already described in Section 3, was used in several clinical studies targeted to different diseases in paediatrics resulting in an amelioration of the health status of children suffering from IBS [138]. In this randomized, double-blind, placebo-controlled, multicentre trial, patients were treated with one sachet (twice in those 12–18 years old) of probiotic mixture containing 4.5×10^{12} bacteria for 6 weeks. The preparation was effective in improving the overall perception of symptoms, the severity and frequency of abdominal pain, abdominal bloating and family assessment of life disruption, leading to a general improving of quality of life in children suffering from IBS.

Miele et al. [139] carried out the first paediatric, randomized, placebo-controlled trial using VSL#3 for the treating of ulcerative colitis (UC). This disorder belongs to the chronic inflammatory bowel disease (IBD) category, has a prevalence of about 100 cases per 100,000 children [140] and occurs as diffuse mucosal inflammation in the colon; it is characterized by periods of remission and relapse episodes, not all the patients tolerate the existing treatment to induce remission for their adverse effects and in 20–30% of paediatric patients failure of the treatment occurs [141]. Since the pathogenesis, beside genetic susceptibility, is linked to compromised immune response and alteration in gut microbiota composition, the idea beyond the study was that 1 year of VSL#3 administration might improve the health status of patients. Subjects with an average age of 10 were supplemented with a weight-base dose of probiotic (4.5×10^{11}–1.8×10^{12} bacteria per day); treated patients showed a significantly higher rate of remission compared to placebo and a significantly lower incidence of relapse within 1 year of follow-up. According to the authors, this success may be related to the use of a mixture of various probiotics, which might have a strong synergic action and to the high bacterial concentration of viable cells contained in the mixture. Furthermore, the probiotic preparation showed to be safe and well tolerable by children with a diagnosis of UC.

The efficacy of VSL#3 in paediatric diseases was also evaluated by Dubey et al. [142], who conducted a double-blind, randomized, placebo-controlled trial treating acute rotavirus diarrhoea in children. VSL#3, containing a total of 9×10^9 bacteria/dose and administered for 4 days, significantly reduced, already on day 2, mean stool frequency and improved stool consistency; these results were also reflected in the lower volume of oral rehydration salts administered in children who received the probiotic. The functional role of VSL#3 was investigated by Sinha et al. [143], who focused on the prevention of neonatal sepsis in low birth weight infants, one of the infections which evolves more rapidly in this paediatric category. The mixture, containing 10^9 bacteria/dose, was administered for 30 days. VSL#3 intake in low birth weight was associated with a non-significant 21% reduction in the risk of suspected sepsis; nevertheless, in the sub-group of infants weighing 1.5–1.99 kg, the reduction of the risk of suspected sepsis was statistically significant, differently from newborns weighing 2.0–2.49 kg. The results of the study allowed to conclude that the intervention may be useful for the most vulnerable subjects of low birth weight.

As infant feeding has a crucial role in developing infant gut microbiota and consequently intestinal immunity, fermented formula milk containing probiotics or prebiotics has been

developed. This approach is aimed at protecting infants from various gastrointestinal disorders by modulating gut microbial composition. The first study that evaluated the effects of a fermented formula milk with *B. breve* C50 and *Streptococcus thermophilus* 065 on the incidence of acute diarrhoea in healthy infants was a randomized, double-blind, placebo-controlled multicentre study, which involved 971 subjects belonging to three different areas of France [144]. The trial was planned to occur in a high risk predicted period for diarrhoea incidence in France (from October to January) and the supplementation lasted 5 months. Although no reduction in the incidence and duration of diarrhoea episodes were observed after the intervention, a lower number of dehydration cases, a lower number of medical consultation cases with fewer oral rehydration solution prescriptions and changes of formula were registered. These outcomes can be considered as indicators of probiotic positive effects on the severity of the disease. According to the authors, these results may be related to the bifidogenic and immunomodulatory properties of fermentation products contained in formula-milk.

5.3. Coeliac Disease

The efficacy of the probiotic mixture containing *B. breve* B632 and *B. breve* BR03 was also shown in children affected by coeliac disease. In this case, the strains were administered as lyophilized powder at a daily dosage of 10^9 CFU of each strain for 3 months in coeliac children on a gluten free diet (GFD). A preliminary important outcome obtained from the intervention was the reduction of pro-inflammatory cytokine TNF-α in blood samples of coeliac children on GFD [145]. The gut microbiota composition was also studied with Next Generation Sequencing (NGS) technology. Unexpectedly, the intervention did not cause changes at the level of the genus or phylum to which the administered probiotics belong but the probiotic acted as a "trigger" element for the increase of *Firmicutes* and the restoration of the physiological *Firmicutes/Bacteroides* ratio that was altered in coeliacs with respect to healthy subjects. Moreover, the intervention restored the normal amount of *Lactobacillaceae* members, reaching almost the same values of healthy subjects [146]. Besides modulating inflammatory condition and gut microbiota composition of coeliac children, *B. breve* supplementation influenced the SCFAs profile; acetic acid had a negative correlation with *Verrucomicrobia*, *Euryarcheota* and particularly *Synergisestes* [147]. Although *Synergisestes* is a minor phylum in human faeces (abundance of 0.01%) of healthy subjects, it was found to have a considerable role for human health because of its negative correlation with TNF-α that may indicate an anti-inflammatory role [148,149]. In the study of Primec et al. [147], the *Synergisestes* phylum clearly confirmed its anti-inflammatory role negatively correlating with pro-inflammatory acetic acid after three months of probiotic treatment.

5.4. Paediatric Obesity

Another pathology in which the gut microbiota may play a notable role is obesity. Although it is accepted that obesity results from disequilibrium between energy intake and expenditure, it is a complex disease and not completely understood. Nowadays, obesity prevalence is spreading especially among children and adolescents and it can be considered a worldwide epidemic. Obesity has been associated with a chronic inflammation that may conduct to insulin resistance [150,151]. Recently, obesity has been associated with a specific profile of the gut microbiota characterized by lower levels of bacteria belonging to *Bacteroides* and *Bifidobacterium* genera compared to that of lean individuals [152]. In addition, bifidobacteria were shown to be higher in children maintaining

normal weight at 7 years old than in children developing overweight and their administration was able to reduce serum and liver triglyceride levels and to decrease hepatic adiposity [153,154]. The mixture of *B. breve* already mentioned (BR03 and B632) was used in a cross-over double-blind randomized controlled trial in order to re-establish metabolic homeostasis and reduce chronic inflammation in obese children [155]. Although the study is still on-going, preliminary results related to the part previous the cross-over demonstrated that a *B. breve* administration in obese children is promising: 8 weeks treatment seems to ameliorate glucose metabolism and could help in weight management by reducing BMI, waist to height ratio and waist circumference [155].

5.5 Allergies

There are increasing evidences that the intestinal microbiota plays an important role in the development of allergic diseases, in particular, low bifidobacteria levels appear to be associated with atopic dermatitis [156]; in the previous section, the potential of *B. breve* in preventing and treating allergy conditions was reported and this impressive role has been confirmed in clinical studies. *B. breve* M-16V revealed to be effective in the treatment of cow's milk hypersensitivity infants with atopic dermatitis [157]. *B. breve*, added to the casein-hydrolysed milk formula at the dosage of 5×10^9 CFU or 15×10^9 CFU per day, increased the proportion of bifidobacteria in the gut microbial composition and ameliorated allergic symptoms by interacting with the immune system and no remarkable dose dependent differences were detected [157]. The synergetic combination of probiotics and prebiotics, known as synbiotic, seems also to be promising in atopic dermatitis treatment. In this regard, Van der Aa et al. [158] studied the effects of a synbiotic mixture on atopic dermatitis in formula-fed infants; the formulation consisted of *B. breve* M-16V at a dose of 1.3×10^9 CFU/100 mL and a mixture of 90% short-chain galactooligosaccharides (scGOS) and 10% long-chain fructooligosaccharides (IcFOS), 0.8 g/100 mL added to formula milk. Although the formulation, administered for 12 weeks, had no effect on atopic dermatitis severity, it significantly modulated the composition and the metabolic activity of gut microbiota, leading to a decrease of pH, high lactate and low butyric levels resembling the metabolic profile of breast-fed infants [159]. The same synbiotic mixture has demonstrated to reduce the prevalence of asthma-like symptoms and the prevalence of asthma medications use after the fulfilment of a 1-year follow-up [160].

The effects of a formulation containing *B. breve* M-16V and *B. longum* BB536 for the prevention of allergies in infants enrolling both mothers and newborns was studied [161]. The formulation was provided as powder daily doses containing 5×10^9 CFU/g of each strain. Pregnant women begun the supplementation 4 weeks before the expected date of delivery and the newborns received the probiotic mixed to water, breast- or formula-milk starting 1 week after birth and continuing for 6 months. The study revealed that prenatal and postnatal supplementation with a bifidobacteria mixture reduced the risk of developing eczema and atopic dermatitis in infants. NGS analyses of newborns' faecal samples showed significant differences of the major intestinal microbial phyla (*Actinobacteria*, *Bacteroidetes*, *Proteobacteria*) of allergic and non-allergic infants at 4 months of age. However, these differences were lost at 10 months of age, highlighting that the microbiota of early stages is particularly important in regulating allergies upset in infants.

5.6. Surgical Procedures

Surgical procedures can also alter gut microbiota composition and functions and disrupt intestinal barrier function, inducing the patient in a condition at risk for infection [162]. A probiotic therapy may be functional for patients improving the immunological function of the intestine and competing against harmful bacteria infection. A pilot study demonstrated that daily administration of *B. breve* Yakult BBG-01(10^9 freeze-dried cells per day) to children younger than 15 years 7 days before surgery until discharge from hospital, simultaneously to intravenous antibiotics postoperatively treatment, reduced the incidence of bacteria in blood samples. Moreover, the intestinal microbial composition was improved by increasing *Bifidobacterium* spp. and reducing potential pathogens such as *Clostridium difficile*, *Pseudomonas* and *Enterobacteriaceae*. Higher concentrations of faecal acetate and lower faecal pH levels were detected in children who received the probiotic 2 weeks after surgery [163]. Improvement of intestinal environment resulting from a perioperative supplementation with the same strain was also observed in neonates undergoing surgery for congenital heart disease [164]. Daily dosage of 3×10^9 CFU of *B. breve* Yakult (BBG-01) was administered starting 1 week before surgery and ending 1 week after the operation; infants who received the probiotic supplement showed significantly higher bifidobacteria levels and lower *Enterobacteriaceae*, *Staphylococcus* and *Pseudomonas* levels in faecal microbiota compared to infants not receiving the supplement. Moreover, probiotic treated infants exhibited significantly higher concentration of total organic acids levels compared to non-treated ones, in particular acetic acid increased immediately and 1 week after surgery; furthermore, the faecal pH tended to decrease with the probiotic intervention.

Kanamori et al. [165] documented in a case-report the efficacy of a synbiotic therapy, consisting in a combination of *B. breve* Yakult (BBG-01), *L. casei* Shirota and galactoolicosaccharides as prebiotic components, in a newborn with short bowel syndrome resulting from a consistent bowel resection performed soon after delivery. Patients affected from this pathology are subjected to an intestinal bacteria overgrowth due to their dilated intestine [166]; this condition can lead to a bacteria translocation in other districts inducing catheter sepsis, compromised carbohydrates fermentation resulting in high level of lactate, with consequent acidosis [167] and a possible incontrollable growth of intestinal pathogens. One year of synbiotic therapy, consisting in 3 g of bacteria (1×10^9 bacteria/g per each strain) and 3 g of prebiotic per day, improved the nutritional state, prior compromised, by increasing the intestinal motility and suppressed the intestinal pathogen overgrowth, in particular *E. coli* and *Candida* spp.

The same synbiotic combination was used as a therapy for refractory and repetitive enterocolitis [168]; this disorder often occurs in paediatric surgery patients and the severe type may be fatal. The 7 recruited patients, having short bowels as a result of surgical resection and suffering from repetitive enterocolitis, were administered with 1 g of probiotic (10^9 bacteria/g) 3 times daily for 36 months. All patients had an altered gut microbial composition prior to the therapy characterized by low levels of anaerobic bacteria and high levels of resident pathogenic bacteria. In spite of the frequent antibiotic treatments to which patients were exposed, the long synbiotic administration was effective in highly increasing bifdobacteria and lactobacilli levels, which were almost undetectable before the supplementation and incrementing faecal SCFAs, inducing a more normal ecosystem profile in the intestine. Moreover, most of patients accelerated their body weight gain and showed increased serum rapid turnover, with a general amelioration of their health status.

With the developing of therapies and surgeries in the field of perinatal and foetal cares, neonate survival outcomes have extraordinary increased; newborns that are subjected to these interventions need prolonged intensive care periods, which include use of antibiotics, respiratory care and restriction of enteral feeding. All these factors may affect the normal microbial gut colonization leading to severe infection and malnutrition [169]. A synbiotic therapy, including *B. breve*, as already observed, could be effective in preventing or correcting an abnormal microbial colonization in intensive care newborns. The same synbiotic therapy, largely and positively tested, including *B. breve* Yakult, *L. casei* Shirota and galactooligosaccharides, was applied to newborns with diagnosis of severe congenital anomalies [169]. The product contained 10^9–10^{10} bacteria/g and was administered immediately after birth via a nasogastric tube, as soon as intestinal feeding was possible, first at a dose of 0.12 g per day in four equal dose and then, when the amount of milk increased, at 3 g per day in three equal doses. As results of the therapy, none of patients manifested enterocolitis, they showed an improvement in their clinical course and reached a body weight gain equivalent to that of normal infants. This last outcome has been hypothesized to be linked to the potential metabolic activity of the administered probiotics to promote liver lipogenesis and fat storage in the peripheral fat tissue contributing to the growth observed in these infants despite the congenital disorders [170].

5.7. Coadjuvant in Chemotherapic Treatment

A condition in which the use of probiotics may have a reliving effect is chemotherapy. The cancer itself and the drug-therapy inducing bone marrow suppression lead to an immunocompromising state in which an infectious could be fatal. Since the main source of infection is endogenous intestinal harmful bacteria [171], a probiotic treatment can certainly benefit the patient's state by not only competing against pathogens for nutrients and attachments sites but also by stimulating gut immunity, producing organic acids and improving transepithelial resistance [172]. A study conducted in 2009 evaluated the effect of *B. breve* Yakult (BBG-01) strain in cancer paediatric subjects, administered with 10^9 freeze-dried cells, corn starch and hydroxipropyl cellulose in 1 g of formulation. The administration was found to be effective in reducing febrile episodes, which may be the only sign of infection and the use of intravenous antibiotics by stabilizing the intestinal microbial composition [173].

An overview in chronological order of *B. breve* applications as a single strain and as a component of a multi-strain/multi-species formulation is reported in Table 1 and Table 2, respectively.

Table 1. Overview of *B. breve* strains applications in *in vitro* studies, mice model and paediatric trials.

***B. breve* strains**	**Reported effect(s)**	**References**
B. breve B632	Strong antimicrobial activity against pathogens, stimulation of mitochondrial dehydrogenase activity of macrophages, stimulation of proinflammatory cytokines production in *in vitro* study	[62]
B. breve BR03	Inhibition of the growth of 4 *E. coli* biotypes in *in vitro* study	[64]
B. breve B632 + *B. breve* BR03	Reduction of total faecal coliforms in healthy children	[65]
	Reduction of pro-inflammatory TNF-α in blood samples of coeliac children	[145]
	Reduction of minutes of daily crying in healthy infants	[129]
	Restoration of the healthy percentage of main gut microbial components in coeliac children	[146]
	Improvement of glucose metabolism and weight management in obese children	[155]
	Reduction of daily vomit frequency, daily evacuation, improved stool consistency, protection against developing metabolic disturbance in healthy infants	[130]
	Modulation of faecal SCFAs profile in coeliac children	[147]
B. breve Yakult (BBG-01)	Anti-infective activity against Shiga-toxin-producing *E. coli* in mice model	[69]
	Reduction of febrile episodes and use of intravenous antibiotics in cancer paediatric subjects	[173]
	Improvement of composition and metabolic activity of gut microbiota and reduction of incidence of bacteria in blood in paediatric surgery subjects	[163]
	Increased defecation frequency, improvement of stool consistency, frequency episodes of faecal incontinence and abdominal pain in constipated children	[133]
	Stimulation of anti-inflammatory IL-10-producing CD4+T cells in mice model	[83]
	Improvement of composition and metabolic activity of gut microbiota in paediatric surgery infants with congenital heart disease	[164]
B. breve YIT4064	Stimulation of anti-influenza virus hemagglutinin IgA production by Peyer's patch cells in mice model	[75]
	Stimulation of antigen-specific IgG production against pathogenic antigens in mice model	[74]
B. breve UCC2003	Reduction of *Citrobacter rodentium* gut colonization in mice model	[76]
B. breve NCC2950	Induction of REGIII-γ expression in mice model and REGIII-α in *in vitro* study	[78]
B. breve MRx0004	Reduction of pro-inflammatory cytokines and lung neutrophil and eosinophil infiltration in severe asthma mice model	[79]
B. breve M-16V	Improvement of allergic symptoms associated to cow's milk hypersensitivity in infants	[157]

	Immunomodulation activity by increasing TGF-β1 in preterm infants	[122]
	Reduction of infections and mortality for NEC in extremely and very low birth weight infants	[118]
	Reduction of faecal butyric acid in extremely and very low birth weight infants	[121]
	Reduction of total IgE, OVA-specific IgE and OVA-specific IgG in mice model	[80]
	Protection against developing of whey allergy symptoms in model mice	[81]
	Reduction of infections and sepsis incidence in extremely and very low birth weight infants	[124]
	Improvement of composition and metabolic activity of gut microbiota in infants with atopic dermatitis	[158]
	Reduction of asthma-like symptoms prevalence and asthma medication use prevalence in infants with atopic dermatitis	[160]
	Shifted gut microbiota towards a healthy profile in preterm infants	[111]
	Low incidence of NEC (≥stage II) in very low birth weight infants	[112]
	Partially protection against developing skin reaction due to cow's milk allergy, increased caecal content of butyrate and propionate and increased antimicrobial IL-22 expression in mice model	[82]
B. breve B-3	Suppression of epididymal fat and body weight gain in mice model with diet-induced obesity	[84,85]
B. breve 1205	Amelioration of anxiety condition and general metabolism in mice model	[88]
B. breve A1	Prevention of cognitive decline in Alzheimer disease and reduction of neural inflammation in mice model	[89]
B. breve NCIMB 702258	Increased CLA isomer (*c*9, *t*11), EPA and DHA in adipose tissue and reduced proinflammatory cytokines in mice model	[96]
B. breve YIT4010	Reduced abdominal symptoms and improved weight gain in preterm infants	[109]
	Establishment of beneficial gut microbiota and prevention of infections in preterm infant	[110]

Table 2. Overview of applications of *B. breve* strains combined to other bacterial strains in paediatric trials.

***B. breve* strains**	**Probiotic mixture**	**Reported effect(s)**	**References**
B. breve M-16V	*B. breve* M-16V *B. longum* BB536	Reduction of developing eczema and atopic dermatitis in infants	[161]
	B. breve M-16V *B. infantis* M-63 *B. longum* BB536	Reduction of abdominal pain prevalence and frequency, improvement of quality of life in IBS children	[134]
B. breve Yakult (BBG-01)	*B. breve* Yakult *L. casei* Shirota	Improvement of composition and metabolic activity of gut microbiota and of overall health status in infants with short bowel syndrome	[165,168]
		Prevention of enterocolitis, improvement of body weight and clinical course in infants with congenital disorders	[169]
	B. breve Yakult *L. casei*	Reduction of NEC incidence and improvement of intestinal motility in infants	[125]
B. breve C50	*B. breve* C50 *S. thermophilus* 065	Reduction of number of dehydration cases and medical consultation cases in children exposed to risk of developing acute diarrhoea	[144]
B. breve DSM 24732	VSL#3	Reduction of stool frequency and improving of stool consistency in children with acute rotavirus diarrhoea	[142]
		Manifestation of high rate of remission and low incidence of relapse in UC children	[139]
		Improvement of symptoms, severity and frequency of abdominal pain and bloating and family assessment of life disruption in IBS children	[138]
		Reduction of the risk of suspected sepsis in most vulnerable very low birth weight infants	[144]

6. *B. breve* Administration in Adults: A Short Outcome

The use of *B. breve* has been largely investigated in paediatric scenery and its therapeutic role has been strongly supported by significant and solid outcomes; its use is not limited to paediatric supplementation but it is also involved in improving health condition in briefly outlined.

Minami et al. [174] investigated the use of *B. breve* B-3 at a daily dosage of 5×10^{10} CFU/capsule for 12 weeks in adults with a tendency for obesity. A significant decrease of the fat mass and an amelioration of blood parameters were observed, in particular a significant reduction of γ-glutamyltranspeptidase (γ-GTP), a marker used to evaluate liver injury and high-sensitivity protein C-reactive (hCRP), a marker used to evaluate the inflammatory reaction, were detected.

Interestingly, a significant negative correlation between the value of fat mass and 1,5-anhydroglucitol, a marker that closely reflect short-term glucose status and glycaemic variability, was recorded suggesting the potential role of *B. breve* in the improvement of diabetes.

Ishikawa et al. [175] showed the effects of one year of *B. breve* Yakult treatment, in association with galactooligosaccharides as prebiotic, in patients diagnosed with UC. The probiotic, containing 10^9 CFU/dose of freeze-dried powder, was administered immediately after every meal 3 times a day and the prebiotic, at a dosage of 5.5 g, was administered once a day. The synbiotic intervention improved the endoscopic score by decreasing the values of severity mucosa damage [176] and reduced the level of myeloperoxidase, which is secreted by neutrophils and macrophages accumulated in the inflamed lesions and positively correlated with the disease severity [177]. Regarding gut environment results, the synbiotic treatment significantly reduced *Bacteroidaceae* counts and faecal pH, which may be connected to an increment of faecal SCFAs.

An interesting relationship was evaluated by Kano et al. [178]: since a Japanese 2007 survey evidenced that women who suffer from abnormal bowel movements also showed skin disorders, they conducted a double-blind, placebo-controlled, randomized trial to investigate the effects of probiotic and prebiotic fermented milk on skin of healthy adult women. The fermented milk contained galactooligosaccharides, polydextrose, *B. breve* Yakult, *Lactococcus lactis* and *S. thermophilus* at a daily dose of 6×10^{10}, 5×10^{10}, 5×10^{10} CFU/100 mL of milk, respectively. The synbiotic intake, which lasted 4 weeks, resulted to prevent hydration level decreases in the stratum corneum. The intervention increased cathepsin L-like protease activity, which can be considered as an indicator of keratocyte differentiation, as proteolysis of cathepsin L activates transglutaminase 3, which plays an important role in the *stratum corneum* formation [179]. Moreover, the administration reduced phenol levels in serum and urine and since the production of phenols is inhibited at low intestinal pH, an increase of intestinal organic acid levels might be occurred after the treatment.

The probiotic preparation VSL#3 has been extensively used for the treatment of IBD in adulthood. Brigidi et al. [180] investigated the effects of 20 days VSL#3 administration in patients with diarrhoea predominant-IBS or functional diarrhoea; the probiotic intake caused changes in gut microbiota composition with a significantly increase of total lactobacilli, total bifidobacteria and *S. thermophilus*, which are component of VSL#3. The treatment led also to an improvement of some enzymes functions, whose actions are compromised in IBD, by reducing urease activity, whose products usually allow pathogenic bacteria to survive in the gastrointestinal tract and contribute to mucosal tissue damages [181] and by increasing β-galactosidase activity, which is involved in the metabolism of unabsorbed carbohydrates. Pronio et al. [182] confirmed the positive role of VSL#3 upon treatment of patients undergoing ileal pouch anal anastomosis for ulcerative colitis. The probiotic intervention reduced signs and symptoms of inflammation inducing a significant expansion of cells associated to an improvement of the inflammatory condition of the pouch mucosa. An interesting microbial outcome was evidenced by Kühbacher et al. [183]: the UC remission maintained by VSL#3 administration was accompanied by a higher bacterial diversity actually not related to the probiotic intake. However, the increase of bacterial diversity may represent a therapeutic mechanism that supports the VSL#3 activity in maintaining UC remission. Bibiloni et al. [184] showed that 6 weeks administration with the probiotic mixture improved UC remission and response in patients not responding to traditional therapy. Since VSL#3 has been demonstrated to maintain remission in UC patients intolerant or allergic to 5-aminosalicylic acid (5-

ASA), known also as mesalazine [185], Tursi et al. [186] demonstrated the efficacy on UC of another therapeutic combination: VSL#3, in association with balsalazide, 5-ASA prodrug, was shown to be significantly superior to balsalazide alone and to mesalazide in the treatment of active mild-to-moderate UC. One of the key points of the study is the low dosage of balsalazide used (2.25 g/day), usually not effective in reducing UC symptoms and inducing remission. Therefore, the low dosage appeared to be effective only in combination with VSL#3. In this regard, a more recent study, involving a larger number of patients, highlighted the superior ability of VSL#3 to improve relapsing mild-to-moderate UC when added to standard UC treatment with respect to patients on standard treatment only, confirming the potential synergic action exerted by standard UC pharmacological treatments and VSL#3 [187]. The reason for this synergic action may be a combined effect of the chemotherapic on the disease and of the probiotic on the general well-being of the host. Clinical studies proved that this probiotic mixture was particularly effective in the treatment of IBD, improving abdominal pain duration and distention severity score in patients suffering from IBS [188]. Moreover, it was effective in clinical condition of diarrhoea-predominant IBS subjects [189,190].

7. Conclusions

This review has outlined the large number of cases in which *B. breve* strains, mainly as single strains but also in combination with other *Bifidobacterium* species or *Lactobacillus* strains, are used for therapeutic and prevention purposes and/or to prevent further complications of the disease in the paediatric sector. The analysis of the outlined results allows to conclude that, whereas *in vitro* or animal-model study are performed with a large number of different *B. breve* strains, clinical studies are performed with a restricted number of strains (mainly *B. breve* YIT4010, M-16V, the associations B632/BR03 and Yakult BBG-01). Therefore, there is the opportunity of expanding the potentialities of the strains used in clinical studies on the basis of the positive results obtained in pre-clinical studies and, therefore, more opportunities for a further development of "therapeutic microbiology." A second interesting aspect outlined in this review is the frequent association of the *B. breve* administration with traditional chemotherapeutic treatment. This is particularly important in the treatment of very serious diseases in which stopping the traditional therapies may be considered risky for the patient. The probiotic can act as a supplement to prevent complication and improve the general health status of the patient. We are all confident that the improvement in the "therapeutic microbiology" sector will be a great aid to medical approach in the near future.

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Paper 2

Three-month feeding integration with *Bifidobacterium* strains prevents gastrointestinal symptoms in healthy newborns

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Abstract

Infantile functional gastrointestinal disorders are common in the first months of life. Their pathogenesis remains unknown although evidences suggest multiple independent causes, including gut microbiota modifications. Feeding type, influencing the composition of intestinal microbiota, could play a significant role in the pathogenesis. Previous studies supported probiotic supplementation success against colics, however mainly *Lactobacillus* spp. were tested. The aim of this study was to evaluate the effectiveness against functional gastrointestinal disorders of a *Bifidobacterium breve* based probiotic formulation including in the study both breastfed and bottle-fed subjects.

268 newborns were enrolled within 15 days from birth. 155 of them effectively entered the study and were randomized in probiotic and placebo group, receiving the formulation for 90 days. The probiotic formulation consists of a 1:1 mixture of 2 strains of *B.breve* prepared in an oily suspension and administered in a daily dosage of 5 drops containing 10^8 CFU of each strain.

Absolute quantification of selected microbial groups in the faeces was performed using qPCR. Anthropometric data, daily diary minutes of crying, number of regurgitations, vomits and evacuations, and colour and consistency of stools were evaluated before and after treatment.

The study confirmed the positive role of breast milk in influencing the counts of target microbial groups, in particular the bifidobacteria community. No adverse events upon probiotic administration were reported, suggesting the safety of the product in this regimen. *B. breve* counts increased significantly in all administered newborns ($p<0.02$). The study demonstrates that a 3 months treatment with *B. breve* strains in healthy breast-fed newborns helps to prevent functional gastrointestinal disorders, in particular reducing 56% of daily vomit frequency ($p<0.03$), decreasing 46.5% in daily evacuation over time ($p<0.03$) and improving the stool consistency (type 6 at the Bristol Stool chart instead of type 5) in those at term ($p<0.0001$) with respect to those in placebo. Moreover, a significant reduction (8,65 vs 7,98 LogCFU/g of feces, $p<0.03$) of *B.fragilis* in the bottle-fed group receiving the probiotic formulation was observed.

Keywords: probiotic, *Bifidobacterium breve*, infant colic, functional gastrointestinal disorders, breastfeeding, bottle-feeding, microbiota

1. Introduction

Infant colic is a common disorder in the first 3 months of childhood that affects up to 30% of newborns and is characterized by paroxysmal, excessive, incontrollable crying without identifiable causes (1). Wessel et al. (2) gave the first definition of this disorder as a condition of crying or fussing that lasts more than three hours per day, more than three days per week. For a clinical purpose, the Rome IV consensus group (3) recently revised the diagnostic criteria including the age of newborn (< 5 months), giving less important to the amount of crying considering instead the prolonged and unsoothable character of the crying episodes as well as irritability that cannot be prevented or resolved by caregivers. Symptoms, such as flushing of the face, meteorism, thighs flexion and flatulence, begin in the second week of life, in both breastfed and formula-fed infants, and usually resolve spontaneously over time (4).

Infant colic represents a serious problem for the family, because caregivers have difficulties in dealing with these incontrollable crises often resulting in stress and concerns; a prospective European multicenter study carried out by Vik et al. (5) revealed that infantile colic and prolonged crying are associated with high maternal depression scores. Similarly, regurgitation, vomit and constipation frequently require a pediatrician visit during the first 6 months of life and are often responsible for feeding changes, and use of medical treatments (6,7). Moreover, several consequences were associated to the presence of colics in the early stage of life: children with a history of colics have a higher prevalence of functional gastrointestinal disorders later in life (8) and children with migraine were more likely to have experienced infantile colic than those without migraine (7). Therefore, an effective preventive strategy against functional gastrointestinal disorders is envisaged.

Despite forty years of research, the aetiology of colic crises and other functional gastrointestinal disorders has not been fully clarified. It has been suggested that a number of behavioral factors (psychological and social), nutritional factors (food hypersensitivity or allergy), intestinal dysmotility and low grade intestinal inflammation can contribute to its occurrence (6,9). Being a typical disorder of the gastrointestinal tract, it is not surprising that imbalance in the gut microbiota composition has been suggested to play a role in the pathogenesis of these conditions. The gut microbiota has a very close relation with the host contributing to the normal human physiology: it can provide a barrier for colonization of pathogens, synthesize vitamins and other beneficial compounds and stimulate the immune system (10). The neonatal period is a crucial stage for gastrointestinal colonization, a balanced composition of the gut microbiota resulting in a positive effects on the host health (11). Colicky infants have a reduced fecal-bacterial diversity and stability, compared to the healthy ones. They also show a higher prevalence of Gram negative bacteria, especially coliforms, and a reduced abundance of beneficial bacteria, such as lactobacilli and bifidobacteria (12).

Diet has a dominant role in shaping the gut microbiota, therefore the type of feeding in newborns has a certain impact on the assessment of the intestinal microbial groups. Remarkable differences were shown by Lee et al. (13): Actinobacteria was the predominant phylum in breastfed newborns, followed by Firmicutes and Proteobacteria; in contrast, in formula-fed infants, the proportions of Actinobacteria and Firmicutes were similar, followed by Proteobacteria. In addition, the gut microbiota of formula-fed infants contains a significant amount of the genera *Escherichia*, *Veillonella*, *Enterococcus* and *Enterobacter*, whereas the content of *Lactobacillus* was low. The same work reports that the main genera in both breast- and formula-fed infants is *Bifidobacterium*,

but the proportion resulted significantly higher in breastfed infants. The study of Mazzola et al. (14) also showed a reduced *Bifidobacterium* spp. count in mixed-fed infants (fed with at least 50% formula milk) with respect to breastfed. On the contrary, a differential representation of the genus *Bifidobacterium* was not detected in breastfed infants compared to formula-fed, although differences in the gut microbiota were observed in the two groups (15). Moreover, these studies detected lower bacterial richness and diversity in breastfed, probably for the presence of unique oligosaccharides in breast milk, which serve as selective metabolic substrates for a limited number of gut microbes (16).

Feeding type, influencing the composition of intestinal microbiota, could play a significant role in the pathogenesis of infant colic although after the first year of life these differences are lost (17). A recent study focused on colicky and non-colicky formula-fed infants, performed using FISH as bacterial counting technique, revealed a lower concentration of total bacteria and a higher abundance of Enterobacteriaceae in colicky formula-fed infants (18).

Several studies support the use of probiotics as therapeutic or preventive agents against various diseases, in particular enteric disorders but also human pathology which are not apparently linked to the microbial gut composition, such as allergies and autoimmune diseases (19,20). A treatment with probiotics, whose beneficial effects on the gut microbiota disorders and on human health are well known, may have a protective effect from gastrointestinal disorders including colics and reduce the symptoms associated, leading to a correct microbial colonization in early infancy, when the gut microbiota is still in a period of adjustment.

Many studies have focused on the administration of *Lactobacillus reuteri* DSM 17938 as probiotic for the prevention or reduction of symptoms of functional gastrointestinal disorders, including colic, regurgitation, vomit and constipation with successful results (6,21,22). In particular, Savino et al. (23) evidenced a lower number of anaerobic Gram negative bacteria, enterobacteriaceae and enterococci in colicky newborns that received *L. reuteri* compared to no treated babies. However, other *Lactobacillus* species, such as *L. delbrueckii* subsp. *delbruekii* DSM 20074 and *L. plantarum* MB 456, have shown inhibitory activity against gas-forming coliforms and they have the potential of being used in the management of infant colic (24). Differently, the administration of bifidobacteria for the treatment of these intestinal disorders remains scarcely investigated, although their role in the healthy newborn gut microbiota has been demonstrated as reviewed by Di Gioia et al. (25). A previous *in vitro* study described the capability of some strains belonging to *Bifidobacterium* genus, including *Bifidobacterium breve* strains, of inhibiting *in vitro* the growth of pathogens typical of the infant gastrointestinal tract including coliforms isolated from colicky newborns (26). Other studies demonstrated the efficacy of a *B. breve*, for the treatment of different infant diseases: Li et al. (27) showed the usefulness in promoting the colonization of *B.breve* and the formation of a normal intestinal biota in low birth weight infants, Wada et al (28) described beneficial effects of this species in immunocompromised pediatric patients on chemotherapy. Moreover, recent studies have evidenced the effectiveness of *B.breve* to reduce the risk of necrotizing enterocolitis in preterm infants (29, 30). In addition, as not deeply explained in the manuscript, the two strains *B.breve* B632 and BR03 have been investigated for their capability of colonizing human intestine, stimulating the immune response, competing against pathogens and their safety assessments have been also demonstrated (26, 31, 32). A recent study also showed the capability of *B. breve* strains, used as probiotic for children with coeliac disease, to act as a "trigger" element for the increase of other beneficial bacterial genus or phylum, like Firmicutes (33).

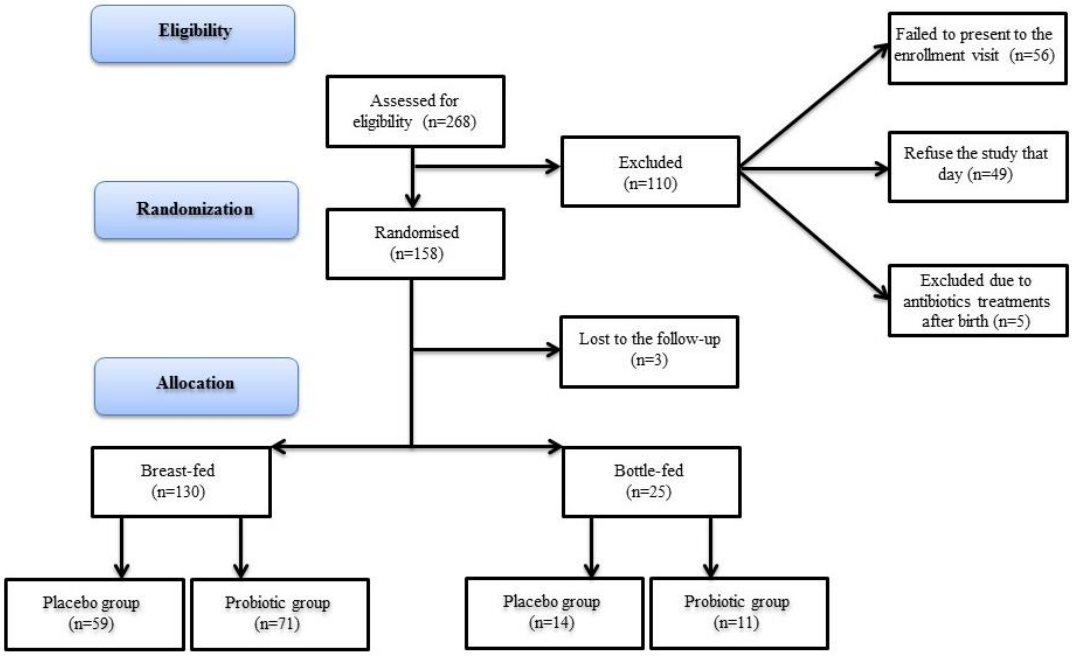
The aim of this study was to describe the effectiveness of a *B. breve* based probiotic formulation administered both to breast-fed and bottle-fed newborns in: 1) shifting the counts of targeted fecal microbial groups; 2) the prevention of colic symptoms and functional gastrointestinal disorders in a cohort of healthy newborns.

2. Materials and Methods

2.1. Study design and samples collection

This was a double-blind, randomized, placebo-controlled clinical trial (NCT03219931) approved by the Ethical Committee of the Maggiore della Carità Hospital (CE 63/13). The newborns were enrolled at the Department of Medical Sciences, Division of Paediatrics, University of Piemonte Orientale "A. Avogadro" in a period from November 2013 to September 2016. Newborns were recruited at birth and enrolled within 15 days from birth during the first visit (T0). Informed consent was obtained by parents at the enrolment, in accordance with the local Ethics Committee and Helsinki criteria. Patients were asked to perform a second visit (T1) after 90 days of treatment. The number of newborns assessed for eligibility (268), randomized (155) and allocated to the placebo or probiotic group is shown in Figure 1. They were recruited if healthy within 15 days from birth and born adequate for gestational age. Exclusion criteria were: 1) twin neonates; 2) treatments with any type of drug within the enrolment; 3) treatments with probiotics; 4) smoking mothers; 5) family history for congenital diseases; 6) history of prolonged jaundice. No specific dietary restrictions during lactation were recommended to the mothers, with the exception of other products containing probiotics. Patients were randomized using a computer-generated allocation sequence in Placebo or Probiotic group (1: 1). The study personnel and parents were masked to the study group allocation. The original idea of the study was to recruit an equal number of breast-fed and bottle-fed newborns but, considering the difficulties in the enrolment in bottle-fed ones, we decided to go on with a different number of newborns belonging to the two groups (Figure 1).
The Probiotic group received a commercial probiotic formulation Bifibaby ® (Probiotical S.p.A., Novara, Italy) containing *B. breve* for 90 days (T1) and the Placebo group received a placebo formulation for the same period. Probiotic formulation was a 1:1 mixture of 2 strains, *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) prepared in an oily suspension, administered in a daily dosage of 5 drops containing 10^8 CFU of each strain. Placebo was prepared with the same excipients without probiotic strains using an identical form of package.

Figure 1. Study flow diagram.



2.2. Clinical monitoring

Delivery and birth data were collected during the first visit. Anthropometric data (weight, height, head circumference) and type of feeding information were collected at both the first (T0) and second visit (T1).

Parents were asked to record on a daily diary minutes of inconsolable crying according to a validated questionnaire (34). They also recorded daily number of regurgitations, vomits, and evacuations, and colour and consistency of stools. The Bristol Stool Form Scale for children was given to parents (35). Colic was diagnosed according to the Rome IV consensus group (3).

Parents were also asked to report any adverse event (in particular constipation, vomit, allergic reactions, illness), treatments, number and type of infections, or abdominal pain occurred during the trial period. The adherence was monitored by biweekly phone calls, counting empty vials, and checking daily dairies.

2.3. Stool samples collection

Faecal samples of newborns were collected twice, on enrolment (T0) and at the end of the intervention with probiotic/placebo (T1). The analysed groups were therefore: Probiotic T0, Placebo T0, Probiotic T1, Placebo T1). Faecal samples were frozen immediately after collection at –80 °C, in numbered screw-capped plastic containers, until they were processed for DNA extraction. Researchers performing DNA extraction and molecular analyses (qPCR) were blind to the group identity of patients (Probiotic or Placebo group).

2.4. DNA extraction from faecal samples

DNA was extracted from 200 mg of faeces (preserved at -80 °C after collection) using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) with a slight modification of the standard protocol: an supplementary incubation at 95 °C for 10 min of the stool sample with the lysis buffer was added to enhance the bacterial cell rupture (36). Extracted DNA was stored at -80 °C. The purity of DNA was determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite®200 PRO NanoQuant, Tecan, Mannedorf, Switzerland) and the concentration was evaluated by Qubit® 3.0 Fluorometer (Invitrogen, Life Technologies, CA, USA).

2.5. Absolute quantification of selected microbial groups using quantitative PCR (qPCR)

Quantification of selected microbial groups or species usually monitored in studies related to infants (36,37), i.e. *Bidobacterium* spp., *Lactobacillus*spp., *Bacteroides fragilis* group (comprising the most abundant species in human *B. fragilis, B. distasonis, B. ovatus, B. thetaiotaomicron, B. vulgatus*), *Bifidobacterium breve, Clostridium difficile, Escherichia coli* and total enterobacteria, was performed with real-time PCR on DNA extracted from stool samples. The assays were carried out with a 20 μL PCR amplification mixture containing 10 μL of Fast SYBR® Green Master Mix (Applied Biosystems, Foster city, CA, USA) optimized concentrations of primers (Table 1 and 2), molecular grade H_2O and 2 μL DNA obtained from faecal samples at a concentration of 2.5 ng/μL. *B. breve* analysis was performed using a TaqMan assay containing 12.5 μL of Universal TaqMan master mix (Applied Biosystems, Foster city, CA, USA) 300nM of each primers and 100 nM of probe labeled with the 5' reporter dye 6-carboxyfluorescein and the 3'quencher NFQ-MGB (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The number of PCR cycles was 40.

The primer concentrations were optimized through primer optimization matrices in a 48-well plate and estimating the best Ct/ΔRn ratio. The different primers were also checked for their specificity utilizing the database similarity search program nucleotide-nucleotide BLAST (38). Moreover, to evaluate the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification. The data obtained from the amplification were then converted to obtain the number of bacterial (Log CFU/g faeces) in accordance with the rRNA copy number available at the rRNA copy number database (39). Standard curves were constructed using 16S rRNA PCR products of type strains of each target microorganism; the standard microorganisms used were *Bifidobacterium breve* ReO2, *Lactobacillus plantarum* ATCC 14917, *Bacteroides fragilis* DSM 2151, *Bifidobacterium breve* B632 DSM 20213, *Clostridium sporogenes* ATCC 319, *E.coli* ATCC 8739. PCR products were purified with a commercial DNA purification system (NucleoSpin® Extract II kit, MACHEREY-NAGEL GmbH & Co. KG, Germany) and the concentration measured spectrophotometrically at 260 nm. Serial dilutions were performed and 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 copies of the gene per reaction were used for calibration. Sample reactions were conducted in triplicate, with a negative control per each reaction.

Table 1. Primer sequences and qPCR conditions used in the different assays.

Microorganism target	Primer	Sequence (5'-3')	Amplicon length(bp)	References
Escherichia coli	Eco-F	GTTAATACCTTTGCTCATTGA	340	(40)
	Eco-R	ACCAGGGTATCTAATCCTGTT		
C. difficile	Cdiff-F	TTGAGCGATTTACTTCGGTAAAGA	114	(41)
	Cdiff-R	TGTACTGGCTCACCTTTGATATTCA		
Bifidobacterium spp.	Bif-F	TCGCGTCYGGTGTGAAAG	243	(42)
	Bif-R	CCACATCCAGCRTCCAC		
Lactobacillus spp.	Lac-F	GCAGCAGTAGGGAATCTTCCA	349	(43)
	Lac-R	GCATTYCACCGCTACACATG		
B. fragilis group	Bfra-F	CGGAGGATCCGAGCGTTA	92	(37)
	Bfra-R	CCGCAAACTTTCACAACTGACTTA		
B. breve	F _IS	GTGGTGGCTTGAGAACTGGAT AG	118	(44)
	R_IS	CAAAACGATCGAAACAAACACTAAA		
	P_IS	TGATTCCTCGTTCTTGCTGT		
Enterobacteria	Ent-F	ATGGCTGTCGTCAGCTCGT	385	(45)
	Ent-R	CCTACTTCTTTTGCAACCCACTC		

Table 2. qPCR amplification protocols and primer concentrations

Taget Bacteria	Initial denaturation	Denaturation	Annealing t (°C)	N. cycles	Fw	Rev
E.coli						
Eco-F/Eco-R	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	400 nM	400 nM
C.difficile						
Cdiff-F/Cdiff-R	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	250 nM	250 nM
Bifidobacterium spp.						
BifTOT-F/BifTOT-R	95°C – 20sec	95°C - 3 sec	60°C - 35sec	40	200 nM	300 nM
Lactobacillus spp.						
Lac-F/Lac-R	95°C – 20sec	95°C - 3 sec	63.5°C - 30 sec	40	200 nM	200 nM
B. fragilis group						
Bfra-F/Bfra-R	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	300 nM	300 nM
B. breve						
F_IS/R_IS	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	400 nM	400 nM
Enterobacteria						
Ent-F/Ent-R	95°C – 20sec	95°C - 3 sec	60°C - 35sec	40	200 nM	300 nM

2.6. Statistical analysis

Data were expressed as mean ±SD. Skewed variables were log transformed. Daily data were divided in 9 categories representing the mean of 10 consecutive days (from 0 to 90 day).

According to the primary outcome, a sample of 58 individuals per group has been estimated to be sufficient to demonstrate a difference between placebo and probiotics of 0.70 Log CFU/g of Bifidobacteria with a SD of 1.6, a 90% power, and a significance level of 95%, and a drop-out rate of 20% according to published data already available during the protocol design (32). According to the secondary outcome, a sample of 55 individuals per group has been evaluated sufficient to reduce

of 30% the proportion of gastrointestinal disorders (colic, regurgitation, vomit, constipation) with an estimated prevalence of 40%, according to literature (1, 6).
Data of microbial counts were subjected to Shapiro test and Bartlett test in order to verify the normal distribution of data and homogeneity of variances. The baseline characteristics were compared with a Fisher's exact test for categorical variables and a two sample t-test or the Welch's t-test when appropriate for continuous variables. A two-way repeated measure ANOVA was performed to evaluate the time effect, the treatment effect and the interaction effects (model 1) on the dependent variables (minutes of crying, stool characteristics, episodes of vomits and regurgitation, microbial counts). Sum of squares type III was used. The following covariates were also subsequently introduced: sex, type of delivery (vaginal, caesarean, operative), IAP, gestational age, neonatal weight (model 2). Model 3 also included the type of feeding during the 90 days (breast- bottle-, mixed-feeding). Furthermore, in model 2 and 3, weight, length, and head circumference were also corrected for the corresponding variable at birth. All the statistical analyses were performed using R Statistical Software and SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Baseline characteristics of enrolled newborns

At birth, 268 newborns were assessed for eligibility and accepted the study. One hundred and ten did not enter the study because failed to present to the enrollment visit (56), refused the study that day (49) or were excluded due to antibiotic treatments after birth (5). The 158 subjects were assigned randomly to placebo or probiotics. Three of them were lost at the follow-up and were excluded (Figure 1). Of the 155 newborns who entered in the protocol, 130 were breastfed (59 placebo, 71 probiotics) and 25 were bottle-fed (14 placebo, 11 probiotics). 81 were males, and 74 females. Moreover, 139 neonates born by vaginal, 10 by cesarean, and 6 from operative delivery. All the enrolled mothers were healthy without suffering of chronic diseases. Fifteen mothers received an intrapartum antibiotic prophylaxis (IAP). Three mothers have an episode of flu during the study (2 subjects in the breastfeeding group and 1 subject in the formula feeding group). No one mother was treated with antibiotics during lactation.
Table 3 represents clinical data and microbiological fecal counts at baseline in the two groups of allocation (placebo and probiotic neonates). Only *Lactobacillus* spp. counts were higher in the placebo group than in the probiotic group at baseline.
Because it is well known that feeding modulates gut microbial composition as well as clinical presentation also in neonates, we investigated if breast- and bottle-fed babies were different at baseline. Supplementary Table 1 represents clinical data and microbiological fecal counts at baseline in the two groups (breast fed and bottle-fed neonates). Crying time ($p<0.05$) and stool frequency were higher ($p<0.04$) and regurgitation episodes were less frequent ($p<0.05$) in breastfed infants. Total enterobacteria ($p<0.004$), *E. coli* ($p<0.03$), and *B. fragilis* group ($p<0.01$) counts were lower in breastfed than in bottle-fed newborns, also when corrected for confounders (sex, gestational age, neonatal weight, type of delivery, IAP and days of life at the entry date).

Table 3. Auxological characteristics of the whole cohort at baseline (T0) according to the allocation treatment.

	Placebo	**Probiotic**
Gender (M/F)	34/39	47/35
Gestational age (weeks)	39.1±1.2	39.3±1.0
Neonatal weight (g)	3307.9±397.5	3298.5±362.7
Length (cm)	50.1±2.0	50.1±1.7
Head circumference (cm)	34.2±1.4	33.9±1.1
Delivery (V/C/O)	63/7/3	76/3/3
Days of life	10.6±1.9	10.4±2.4
Breast-/Bottle-feeding	59/14	71/11
Daily crying (min)	25.5±28.8	28.8±37.7
Stool frequency	3.8±1.8	4.1±1.9
Regurgitation episodes	1.7±1.8	1.6±1.4
Vomit episodes	0.1±0.2	0.2±0.4

Data are expressed as mean±SD. Abbreviations: C: cesarean; O: operative; V: vaginal.

3.2. Microbiological results in whole cohort after probiotic and placebo administration

Table 4 shows the average crude microbial count obtained from the two groups of samples: probiotic and placebo. This analysis showed a significant increase of *B. breve* counts after 3 months. The other microbial group did not show any significant difference.

Following this first evaluation and considering the different sample dimension of breast- and bottle-fed newborns as well as differences of baseline microbial counts in these two groups, an analysis separating breastfed from bottle-fed newborns was carried out.

Table 4. Mean counts (Log CFU/g of faeces) of different microbial groups analyzed in stool samples of the whole cohort

Target	Probiotic T0	Probiotic T1	Placebo T0	Placebo T1
Bifidobacterium spp.	7.00±1.41	7.51±0.88	6.88±1.14	7.29±1.06
B. breve	4.45±1.85	6.40±1.31*	4.54±1.5	5.33±1.5
Enterobacteria	6.54±1.23	6.38±1.14	6.02±1.4	6.5±1.02
E. coli	6.72±1.93	7.2±1.36	6.35±2.07	7.42±1.24
Lactobacillus spp.	6.56±1.28	5.60±1.23	6.22±1.06	5.28±1.48
B. fragilis group	7.44±2.14	7.62±1.97	6.79±2.19	7.23±2.00
C. difficile	2.66±1.48	2.82±1.50	2.60±1.32	3.06±1.58

*significant changes at t-test ($p<0.02$)

3.3. Data evaluation of probiotic treatment on breastfed newborns

At baseline, the placebo group had less stool frequency ($p<0.03$) and lower enterobacteria counts than the probiotic one ($p<0.01$), also when corrected for confounders (sex, gestational age, neonatal weight, type of delivery, and days of life at the entry date). The marginal means for stool frequency and enterobacteria counts are: 3.2±0.4 vs 4.3±0.3 ($p<0.01$), and 5.86±0.36 vs 6.01±0.38 Log CFU/g, ($p<0.03$), respectively.

The compliance to the treatment was high. All the parents answered to the phone calls. 98.7% of them brought back correctly the empty vials. No adverse events were reported. Three infants (1 in placebo and 2 in probiotic) received simethicone for several days (less than 30 days) due to regurgitations.

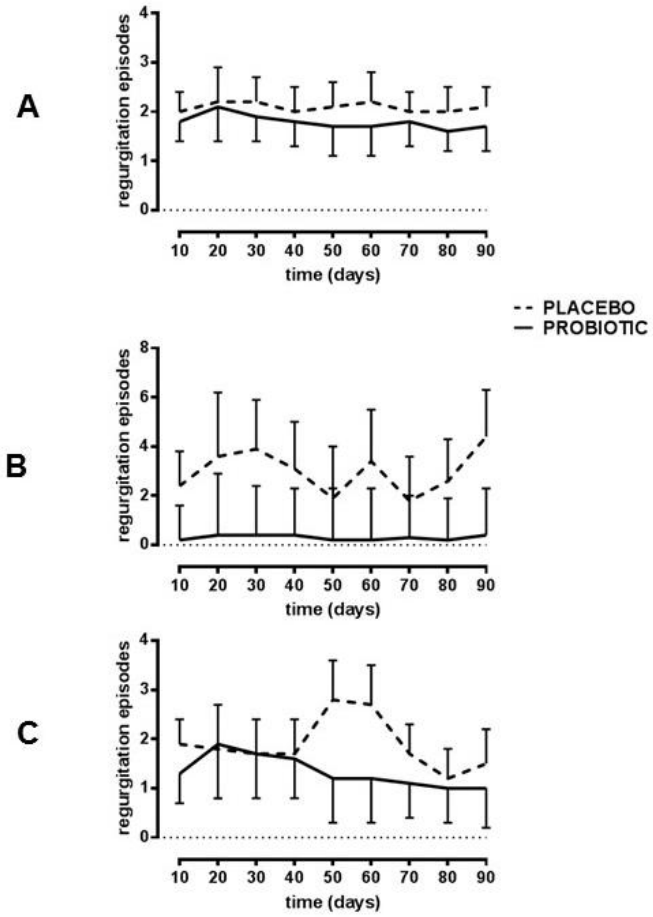
After 90 days, 100 (43 in placebo, 57 in probiotics) of the 130 neonates were still breastfed. In the remaining 30 infants, 7 were bottle-fed (5 in placebo, 2 in probiotic), and the other 23 were in mixed feeding (11 in placebo, 12 in probiotic).

Clinical data. Considering clinical data, minutes of crying ($p < 0.005$) decreased over time without an effect of treatment also in the corrected models.

The number of the evacuation decreased over time ($p<0.0001$), with an effect of treatment in those born after 40 weeks of gestational age ($p<0.03$). Stool consistency was more type 5 at the Bristol Stool Chart ($p < 0.03$) over time. Children born after 40 weeks of gestational age has more frequently type 6 at the Bristol Stool Chart in those in probiotics ($p<0.0001$).

Episodes of regurgitation decreased constantly over time ($p<0.01$) in the probiotic groups diversely by placebo groups accordingly to different type of feeding ($p<0.03$, Figure 2).

Figure 2. Number of daily regurgitations. Probiotic group (continuous line) and placebo group (dotted line). Breastfed newborns **(A)**; Bottle-fed newborns **(B)**; Mixed-fed newborns **(C)**. Data are expressed as marginal mean ± SEM. Data are significant in interaction ($p<0.04$; model 3). The residuals are not homogenous across the groups.



Episodes of vomits decreased significantly with time in the probiotic group but not in the placebo group ($p<0.03$). Moreover, during the 90 days the prevalence of colic infants was similar in the placebo (4 subjects, 6.8%) and probiotic group (6 subjects, 8.5%).

Interestingly, also auxological variables were modified. Infants in probiotics had a lower increase in weight during the study in those born with a cesarean delivery ($p<0.03$; Figure 3), and in those still breastfed or switched to bottle-fed during the study ($p<0.005$). Diversely, infants in probiotics had a higher increase in head circumference in those bottle-fed or with a mixed-feeding ($p<0.01$).

Figure 3. Weight variation in 90 days. Probiotic group (grey bar) and placebo group (black bar). Newborns born by vaginal delivery **(A)**; Newborns born by cesarean delivery **(B)**; Newborns born by operative delivery **(C)**. Data are expressed as marginal mean ± SEM. Data are significant in interaction (p<0.03; model 2). T0: baseline. T1: after 90 days of placebo/probiotic.

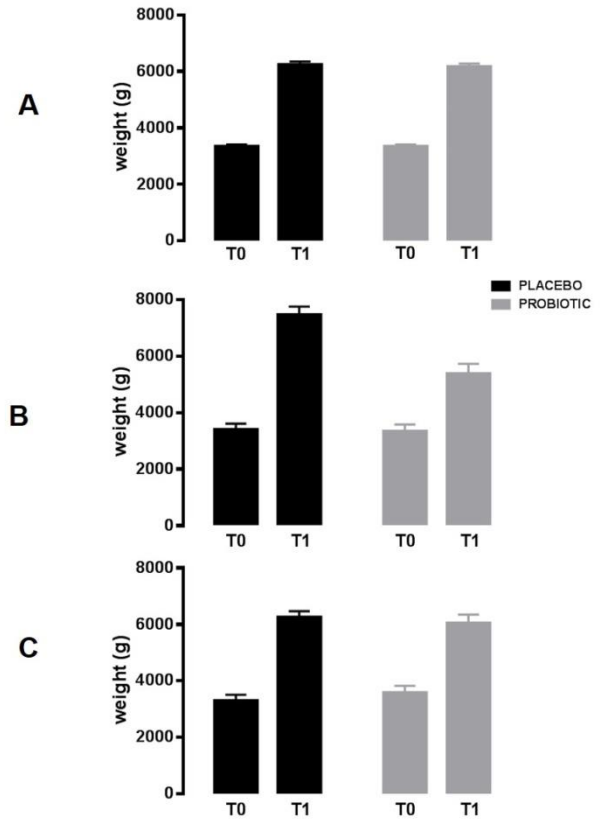


Table 5 describes marginal means of model 1. Supplementary Table 2 describes also corrected models.

Microbial data. Total Enterobacteria ($p < 0.005$), *Bifidobacterium* spp. ($p < 0.001$) and *E. coli* ($p < 0.001$) changed within time, but the significance was lost when corrected for confounders. *C. difficile* did not change.

In those treated with probiotics, *B. fragilis* group members decreased within time in those born vaginally, whereas increased in the other infants ($p < 0.04$). Moreover, *B. breve* increased within time in those treated with the probiotics ($p < 0.04$). Microbiological data are reported in Table 6.

Table 5. Clinical and anthropometric variations in the breast-fed group obtained with multivariable analysis of repeated measure. All values are expressed as marginal means ± standard error.

Target	Probiotic T0	Probiotic T1	Placebo T0	Placebo T1
Crying(min)	25.4±4.5	19.3±2.9	32.1±5.6	20.5±3.7
Stool frequency	4.3±0.2	2.1±0.1	3.9±0.2	2.0±0.1
Stool color	6.0±0.1	5.7±0.1	6.0±0.1	6.0±0.1
Stool consistency	5.8±0.1	5.7±0.1	6.0±0.1	5.6±0.1
Regurgitations	2.0±0.2	1.6±0.2	1.8±0.2	2.0±0.3
Vomits	0.25±0.05	0.11±0.06*	0.10±0.05	0.20±0.07
Weight (g)	3310.9±43.7	3465.6±57.4	3321.5±48.0	3452.2±62.9
Length (cm)	50.1±0.2	51.5±0.2	50.2±0.2	51.5±0.2
HC (cm)	33.9±0.1	34.9±0.1	34.3±0.1	35.0±0.1

Abbreviation: HC, head circumference

*significance in interaction (time*treatment) effect ($p<0.03$)

Table 6. Mean counts (Log CFU/g of faeces) of different microbial groups analyzed in stool samples of breastfed newborns. All values are expressed as marginal means ± standard error.

Target	Probiotic T0	Probiotic T1	Placebo T0	Placebo T1
Bifidobacterium spp.	7.11±0.38	7.91±0.27	6.59±0.34	7.78±0.24
B. breve	4.64±0.50	6.10±0.47*	4.40±0.48	5.86±0.46
Enterobacteria	6.01±0.38	6.55±0.35	5.86±0.36	6.46±0.34
E. coli	6.70±0.57	7.46±0.51	6.40±0.54	7.06±0.48
Lactobacillus spp.	6.22±0.33	5.55±0.33	6.28±0.29	6.27±0.35
B. fragilis group	6.34±0.64	7.33±0.61	6.31±0.61	6.45±0.58
C. difficile	2.70±0.42	2.89±0.48	2.82±0.37	3.01±0.42

*significance in interaction (time*treatment) effect ($p<0.04$)

3.4. Data evaluation of probiotic treatment on bottle-fed newborns

At baseline, placebo group had less *Lactobacillus* spp. counts than probiotic one ($p<0.008$). When corrected for confounders (sex, gestational age, neonatal weight, and days of life at the entry date), the statistical significance was lost, whereas *B. fragilis* group counts were higher in the probiotic group (marginal means are 6.62±0.53 vs 8.62±0.67 Log CFU/g, $p<0.02$).

Clinical data. Considering clinical data in both crude and corrected analysis, no changes were detected in minutes of crying, stool frequency and consistency, episodes of vomits or regurgitations. No infants had colic in both groups. Weight ($p<0.0006$), length ($p<0.01$), and head circumference ($p<0.005$) increased with time without an effect of the treatment.

Microbial data. Total enterobacteria and *E. coli* did not change over time. *Bifidobacterium* spp. ($p < 0.02$) and *C. difficile* increased ($p < 0.04$) with time without a treatment's effect. In those treated with probiotics, *B. fragilis* group ($p <0.03$) decreased and *B. breve* increased ($p <0.03$), respectively with time. Microbiological data are reported in Table 7.

Table 7. Mean counts (Log CFU/g of faeces) of different microbial groups analyzed in stool samples of bottle-fed newborns. All values are expressed as marginal means ± standard error

Target	Probiotic T0	Probiotic T1	Placebo T0	Placebo T1
Bifidobacterium spp.	6.80±0.36	7.56±0.36	6.79±0.30	7.35±0.30
B. breve	4.20±0.35	6.42±0.37 *	4.11±0.31	5.09±0.33
Enterobacteria	7.30±0.41	6.97±0.34	6.75±0.36	7.14±0.30
E. coli	7.91±0.95	7.38±0.62	6.67±0.66	7.51±0.54
Lactobacillus spp.	6.81±0.29	6.00±0.35	6.05±0.26	6.39±0.31
B. fragilis group	8.65±0.61	7.98±1.84 *	7.56±0.56	8.33±0.51
C. difficile	2.54±0.44	3.16±0.52	2.59±0.39	3.35±0.46

*significance in interaction (time*treatment) effect ($p<0.03$)

4. Discussion

The use of bifidobacteria as probiotics in infants is established for some enteric diseases, the most common of which is diarrhea (25). However, although *in vitro* studies support the use of bifidobacteria against gas-forming coliforms (26), no clinical trials have been performed up to now on their use against infant colics. This work was focused on the evaluation of the effects on functional gastrointestinal symptoms, including colics, of integration of the infant diet with a *B. breve* based probiotic formulation.

The study has clearly shown the capability of the administered *B. breve* strains to survive to gastric transit and to reach the neonatal intestine. In fact, although *B.breve* was detected in all fecal samples, a significant increase was shown upon strain administration. In agreement with Lee et al. (13), a reduction of *Lactobacillus* counts was observed in all groups of newborns over time and this

is particularly evident in the probiotic treated group. This could be related to a high ability of *Bifidobacterium* spp. to influence gut microbiota composition, by enhancing the blooming of some species and reducing others, as observed in other studies regarding *Bifidobacterium* administration (25).

Feeding type is known to have a crucial role in shaping the infant intestinal microbiota (25, 46). Our study shows that, at the enrolment, when 7-15 days of breast- or bottle-feeding had already been done, some differences were present in the groups with different feeding type: total enterobacteria and *E. coli* counts were higher in bottle-fed than in breast-fed newborns, also when corrected for confounders. In addition, higher counts of *B. fragilis* were found in bottle-fed infants at the baseline, in agreement with the higher risk of infection generally observed in non-breast fed infants (47). This higher count was also evident after treatment, both in the probiotic and the placebo group, confirming the absolute importance of the starting feeding type in shaping the gut microbiota and, in particular, in reducing Gram negative bacteria amount. However, in bottle-fed infants, the mean counts of *B. fragilis* were higher at the end of the treatment in the placebo group with respect to the probiotic one, thus indicating a possible positive effect of the *B. breve* administration at least before weaning. The increase of *B. breve* is also observed in breastfed newborns not treated with probiotic and this, as already mentioned before, once more highlights the positive role of breast milk in shaping the bifidobacteria community, also considering that *B. breve* is one of the most abundant species in the newborn gut (48). This increase is also supported by the presence of peptides and oligosaccharides in the human milk that provide the stimulation of the growth of bifidobacteria (49).

In addition to the microbial data, this study aims at monitoring the typical gastrointestinal symptoms of colics, i.e. regurgitation, vomit and constipation, all of them difficult-to-handle problems for caregivers. Results obtained from the applied models showed a decreased number of evacuations and an enhancement of stool consistency in breastfed newborns after 90 days of probiotics. In addition, bottle-fed newborns showed an improvement of stool color. These data suggested an improvement in the gastrointestinal transit which can be attributed to the probiotic intake. Moreover, the number of regurgitations and episodes of vomit was reduced after probiotic treatment. Similar results have already been demonstrated with a supplementation of *L. reuteri* (6). The reduction of these symptoms is particularly important because they also reduce parental anxiety and related consequences.

The reduction in regurgitation and vomit was not shown in the group of bottle-fed newborns. This result can be affected by the small size of the bottle-fed group of newborns. The study was not designed to evaluate differences between the two feeding regimens and authors are aware that the bottle-fed group was underpowered to reach the clinical outcomes. However data related to the different feeding should be analyzed separately due to the unexpected significant differences in microbial composition at baseline. On the other hand, those breast-fed at the recruitment who switched to bottle-or mixed-feeding had an improvement with reduction of regurgitation episodes. This is an important achievement also considering that the number of newborns bottle-fed since the beginning of life is generally low as, usually, a starting feeding with mother milk is applied (50).

In this study, daily infant crying time did not show any difference between probiotic and placebo groups in spite of the improvement of the gastric transit due to the probiotic administration. This result is contrasting with other reports in literature. Several causes could be considered, first of all the inaccuracy of the count of minutes of crying through self-report diaries, although validated, in

particular for such a prolonged time. Analysis of any existing tool to monitor daily crying have been demonstrated to be inaccurate, difficult or not validated for a prolonged time of observation (51). Moreover, we evaluated the effects over 3 months, whereas the majority of the studies are related to probiotic treatment no longer than 4 weeks (1, 52). Moreover, other confounding factors may have a role after the second months of life, in particular, if we consider the efficacy on the other gastro-intestinal parameters. Furthermore, the prevalence of colic infants was similar but very low in both placebo and probiotic groups. This is a consequence of considering in the study healthy newborns. Studies including only colic infants are needed in the future.

The main unexpected and interesting result of the study was related to the auxological parameters. Clinical trials on the effect of probiotics on neonatal growth parameters are scarce. In our population, infants born by cesarean section had a lower catch-up growth in weight if treated with the probiotic. This result is of crucial interest in planning further intervention studies. Gut colonization by environmental microorganisms occurs during or immediately after the birth, whereas, in infants delivered by cesarean section, gut colonization is delayed and often altered, in particular modifying *Bifidobacterium* and *Lactobacillus* counts (25). Increasing epidemiological data suggested that children born by cesarean section have an increased risk to develop obesity later in life (53,54). How the genetic background and the environment affect mechanisms that control appetite, weight regulation and metabolic disorders linked to overweight, and the immune education is poorly understood. Gestation, delivery, postnatal nutrition (lactation and weaning) have been identified as critical periods to program the nutritional and hormonal control of the offspring. Some Authors suggest that the sudden modification of the initial conditions may disrupt the physiological process predisposing to certain diseases (55-57) and alterations in the precocious colonization have a role (53,54). Our data suggest that a treatment with *B. breve* strains in the first 3 months of life is able to influence the microbiota composition and this is associated with a concomitant lower weight gain in the population at higher risk of metabolic disturbances in later life. Other authors failed to show changes in weight in neonates treated with other probiotics (52,58). Differences should be secondary to the strains or, more probably, to the timing of the treatment being our protocol designed on three months, differently from the majority of the studies which followed infants for 1 months. In our study, the effect on weight was associated to an increase in head circumference. These data suggest that the probiotic treatment protects against a growth failure, as recently demonstrated for a multi-strain probiotic containing bifidobacteria in very low birth weight children exposed to antibiotics (59).

The main limitation of this study is related to the small sample size of the bottle-fed population. However, this is a consequence of the inclusion criteria (healthy neonates) in a condition in which breastfeeding must be the first choice (50). Furthermore, although we used a validated questionnaire for daily infant crying, the other questionnaires used to record other gastrointestinal symptoms are not validated. On the contrary, the strengths of our study are a treatment prolonged for more than four weeks, the inclusion of neonates not exposed to antibiotics, the evaluation of many confounders, in particular regarding birth and changes in feeding over time.

In conclusion, our study demonstrates that the administered *B. breve* strains can reach the intestine of healthy probiotic newborns, preventing functional gastrointestinal disorders and reducing the precocious weight gain, at least in the absence of antibiotic interferences. No adverse events were reported, suggesting the safety of the product in this regimen. Prospective longitudinal evaluations

should be useful to further investigate if a precocious short treatment in this critical window has also advantages later in life.

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Supplementary Materials

Supplementary Table 1. Auxological characteristics of the whole cohort at baseline (T0) according to the type of feeding.

	Breastfed neonates	**Bottle-fed neonates**
Gender (M/F)	64/66	17/8
Gestational age (weeks)	39.2±1.1	39.1±1.2
Neonatal weight (g)	3315.7±367.4	3236.4±432.7
Length (cm)	50.2±1.8	49.7±1.6
Head circumference (cm)	34.0±1.3	34.0±1.3
Delivery (V/C/O)	119/6/5	20/4/1
Days of life	10.5±2.2	10.6±2.2
Daily crying (min)*	29.2±36.3	19.6±18.6
Stool frequency*	4.1±1.8	3.4±1.8
Regurgitation episodes*	1.6±1.3	2.1±2.5
Vomit episodes	0.1±0.4	0.1±0.3
Bifidobacterium spp. (Log CFU/g)	6.97±1.33	6.79±1.11
B. breve (Log CFU/g)	4.68±1.73	4.15±1.14
Enterobacteria (Log CFU/g)*	6.07±1.31	6.99±1.37
E. coli (Log CFU/g)*	6.15±1.85	7.21±1.41
Lactobacillus spp. (Log CFU/g)	6.48±1.18	6.38±1.02
B. fragilis group (Log CFU/g)*	6.75±2.25	7.96±2.07
C. difficile (Log CFU/g)	2.58±1.45	2.57±1.45

Data are expressed as mean±SD. Abbreviations: C: cesarean; O: operative; V: vaginal.

*significant differences at both Welch's t-test and ANCOVA (corrected for sex, gestational age, neonatal weight, type of delivery, and days of life at the entry)

Supplementary Table 2. Clinical and anthropometric variations obtained with multivariable analysis of repeated measure.

	Model 1			Model 2			Model 3		
	time	treatment	Interaction	time	treatment	Interaction	time	treatment	Interaction
Crying(min)	F: 4.218 p< 0.005	F: 0.236	F: 0.788	F:0.635	F: 1.330	F: 1.330	F:0.563	F: 0.144	F: 0.442
Stool frequency	F: 60.722 p < 0.0001	F: 0.334	F: 0.699	F:0.570	F: 0.228	F: 2.423 p=0.065[a]	F:0.821	F: 0.048	F: 2.513 p<0.04[a]
Stool color	F: 2.41 p < 0.03	F: 0.082	F: 1.323	F:0.844	F: 0.002	F: 2.292 p<0.03[b]	F:0.374	F: 0.045	F: 1.988 p<0.04[b]
Stool consistency	F: 2.410 p<0.01	F: 0.082	F: 1.323	F:1.035	F: 1.375	F: 4.308 p<0.001[c]	F:2.210 p<0.01	F: 0.516	F: 3.983 p<0.001[c]
Regurgitations	F: 2.212 p<0.01	F: 0.062	F: 1.792 P:0.076	F:0.999	F: 0.583	F: 0.240	F:0.983	F: 0.096	F: 2.000 p<0.04[d]
Vomits	F: 1.035	F: 0.477	F: 3.061 p<0.03	F:0.486	F: 4.351 p<0.01	F: 2.177 p<0.02[e]	F:0.178	F: 0.718	F: 2.000 p<0.04[d]
Weight (g)	F: 25.967 p<0.0001	F: 0.001	F: 0.183	F:27.042 p<0.0001	F: 3.431	F: 3.428 p<0.03[e]	F:30.478 p<0.0001	F: 2.630	F: 5.597 p<0.005[d]
Length (cm)	F: 92.391 p<0.0001	F: 0.065	F: 0.005	F:24.272 p<0.0001	F: 0.248	F: 1.106	F:24.878 p<0.0001	F: 0.089	F: 0.356
HC (cm)	F: 70.631 p<0.0001	F: 1.601	F: 2.399	F:20.172 p<0.0001	F: 0.030	F: 0.137	F:23.009 p<0.0001	F: 0.002	F: 4.532 p<0.01[e]

Abbreviation: HC, head circumference.

Model 1. A two-way repeated measure ANOVA was performed to evaluate the time effect, the treatment effect and the interaction effects on the dependent variables (minutes of crying, stool characteristics, episodes of vomits and regurgitation, clinical characteristics).

Model 2. Model 1 plus the following covariates: sex, type of delivery (vaginal, caesarean, operative), IAP, gestational age, neonatal weight

Model 3. Model 1 plus the following covariates: sex, type of delivery (vaginal, caesarean, operative), IAP, gestational age, neonatal weight, feeding during the 90 days (breast- bottle-, mixed-feeding).

Significant interactions in model 2 and 3: a: time*treatment*gestational age*sex; b: time*treatment*sex* type of delivery; c: time*treatment*gestational age; d: time*treatment*type of feeding at visit 2; e: time*treatment; e: time*treatment* type of delivery

Paper 3

Effect of *Bifidobacterium breve* on the intestinal microbiota of coeliac children on a gluten free diet: a pilot study

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Abstract

Coeliac disease (CD) is associated to alterations of the intestinal microbiota. Although several *Bifidobacterium* strains showed anti-inflammatory activity and prevention of toxic gliadin peptides generation *in vitro*, few data are available on their efficacy when administered to CD subjects. This study evaluated the effect of administration for three months of a food supplement based on two *Bifidobacterium breve* strains (B632 and BR03) to restore the gut microbial balance in coeliac children on a gluten free diet (GFD). Microbial DNA was extracted from feces of 40 coeliac children before and after probiotic or placebo administration and 16 healthy children (Control group). Sequencing of the amplified V3-V4 hypervariable region of 16S rRNA gene as well as Q-PCR of *Bidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis* group *Clostridium sensu stricto* and enterobateria were performed. The comparison between CD subjects and Control group revealed an alteration in the intestinal microbial composition of coeliacs mainly characterized by a reduction of the *Fimicutes/Bacteroidetes* ratio, of *Actinobacteria* and *Euryarcheota*. Regarding the effects of the probiotic, an increase of *Actinobacteria* was found as well as a re-establishment of the physiological *Firmicutes/Bacteroidetes* ratio. Therefore, a 3-month administration of *B.breve* strains helps in restoring the healthy percentage of main microbial components.

Keywords: coeliac disease, gluten free diet, probiotic, *Bifidobacterium breve*, intestinal microbiota, qPCR, Next Generation Sequencing

1. Introduction

Coeliac disease (CD) is a chronic gastrointestinal tract disorder showing damages at the small intestine which are hypothetically linked to an autoimmune response caused by gluten ingestion in genetically predisposed subjects. CD in Europe and North America is estimated to affect about 1% of the population, although its incidence in western countries is increasing in the last decades [1,2].. CD is usually chronic but the lifelong adherence to a gluten-free diet (GFD) keeps the disease under control: the small intestine returns to its physiological condition and subsequent tests for CD specific autoantibodies are negative [3,4]. Even if the adherence to a GFD is the only effective solution against CD, patients risk to suffer from an unbalanced nutritional intake and difficulties to adhere to the strict GFD are frequently reported.

The gut microbiota has a very close relation with the host contributing to the normal human physiology. It can provide a barrier for colonization of pathogens, synthesize vitamins and other beneficial compounds and stimulate the immune system. Environmental factors can lead to a disturbance of the microbiota composition, disrupting microbiota-host mutualism and shifting from a condition of homeostasis to a disease-associated profile [5]. In the last decade, CD has been associated to an altered composition of the intestinal microbiota even though studies reported in literature show that there is not a characteristic "coeliac intestinal microbiota" [6]. Some authors evidenced an intestinal dysbiosis in CD patients with active disease characterized by a remarkable reduction in Gram positive bacterial population in duodenal and faecal specimens facilitating the colonization of potentially harmful Gram negative bacteria within the mucosal surface of CD patients [7–9]. In particular, data obtained from duodenal biopsies revealed a reduction in the number of bifidobacteria [10] and changes in species distribution within the *Bifidobacterium* genus have been evidenced by PCR-DGGE [11]. Moreover, symptom free CD patients adherent to a GFD at least for two years did not completely restore the microbiota composition and this condition can lead to a different metabolomics profile [9]. Bacteria belonging to the *Bifidobacterium* genus are well known for their health promoting properties and for their capability of stimulating cells to produce immune molecules and modulating the physiology of gut-associated lymphoid tissue (GALT) [12]. In particular, *in vitro* studies have been focused on the capability of bifidobacteria to increase the IL-10 secretion when co-incubated with mononuclear cells and faecal samples from CD patients [13]. Moreover, a *Bifidobacterium lactis* strain and a probiotic product containing *Lactobacillus* and *Bifidobacterium* strains resulted effective in reducing gliadin induced epithelial permeability through prevention of the toxic gliadin peptide generation during *in vitro* digestion [14–16].

Despite the encouraging data on the potential of probiotic strains, particularly bifidobacteria, *in vivo* studies in patients with CD remain still very scarce. Until now only few studies have taken into account the direct administration of bifidobacteria in subjects affected by CD. Smecuol et al. [17] studied the effects of *Bifidobacterium infantis natren life start* strain in untreated CD patients or rather on a gluten containing diet. Authors found that *Bifidobacterium* administration may alleviate symptoms of untreated CD but it could not modify intestinal permeability. A second study [18] evaluated the administration of *Bifidobacterium longum* CECT 7347 in children on a GFD with newly diagnosed CD and it revealed a reduction of CD3+ T lymphocytes and TNF-α due to probiotic ingestion. To date no studies on CD considered the administration of *Bifidobacterium breve* strains although this species has proved very successful in several paediatric trials regarding necrotizing enterocolitis, immunodeficiency and constipation [19–21].

This work is aimed at the assessment of the impact of the administration of two *Bifidobacterium breve* strains on the gut microbiota composition of coeliac patients compliant to a GFD and, at the same time, it evaluates the difference in the intestinal colonization of coeliac subjects on a GFD for several years with respect to healthy subjects.

2. Materials and Methods

2.1 Study design and samples collection

The study was a double-blinded placebo controlled intervention including 40 patients affected by CD and 16 healthy children for the Control group recruited at a single centre, Department of Paediatrics, University Clinical Centre Maribor in a period from October 2013 to June 2014. Children with CD, aged between 1 to 19 years, were positive to serologic markers for CD and positive for small bowel biopsy, according to ESPGHAN criteria for CD [22]. More details about patients and inclusion/exclusion criteria of the recruiting process are available in Klemenak et al. [23]. The study was registered at https://www.clinicaltrials.gov (registration number: NCT02244047). Patients affected by CD have been randomly allocated into two groups: 20 in the Probiotic group and 20 in the Placebo group. The Probiotic group of patients received an experimental formulation containing *B. breve* for three months and the Placebo group received a placebo formulation for the same duration. Probiotic formulation was a mixture of 2 strains, *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) (1:1), administered as lyophilized powder in a daily dosage of 10^9 CFU of each strain. Placebo was prepared with the same excipients without probiotic strains using an identical form of package. Each package of 2 g powder was mixed with fluids and ingested in the morning breakfast for three months.
Faecal samples of CD patients were collected twice, on enrolment (T0) and at the end of intervention with probiotic/placebo (T1). Members of Control group were sampled only once. Faecal samples were frozen immediately after collection at –80 °C, in numbered screw-capped plastic containers, until they were processed for DNA extraction. Researchers carrying out DNA extraction and molecular analyses (qPCR and sequencing) were blind to the group identity of patients (Control, Probiotic or Placebo group).

2.2 DNA extraction from faecal samples

DNA was extracted from 200 mg of faeces (preserved at -80 °C after collection) were used using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex,UK) with a slight modification of the protocol: an additional incubation at 95 °C for 10 min of the stool sample with the lysis buffer was added to improve the bacterial cell rupture [24]. Extracted DNA was stored at -80 °C. The purity of extracted DNA was determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite®200 PRO NanoQuant, Tecan, Mannedorf, Switzerland) and the concentration was estimated by Qubit® 3.0 Fluorometer (Invitrogen, Life Technologies, CA, USA).

2.3 Preparation of DNA libraries for Illumina MiSeq sequencing

The sample subjected to sequencing belonged to the following groups: 20 Probiotic group T0, 20 Probiotic group T1, 20 Placebo group T0, 20 Placebo group T1 and 16 Control group (Figure 1).
They were processed to amplify and sequence the V3-V4 region of the 16S rRNA gene. The amplicons, approximately 460 bp in length, were generated using the forward and reverse primers, respectively:
5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3'
5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3'
already used in [25].
Each 25 µl PCR reaction contained 12.5 µl of HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA), 5 µl of each primer (0.2 µM) and microbial DNA (5 ng/µl). PCR amplification was performed using the following program: heated lid at 110 °C, 95 °C for 3 min followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by a final elongation step at 72 °C for 5 min. PCR products were cleaned using the AMPure beads XP purification system (Beckman Coulter, UK) following Illumina 16S Ribosomal RNA Gene Amplicon instructions. Illumina sequencing adapters and dual-index barcodes were added to amplicons using the Nextera XT index kit (Illumina, San Diego, CA). The following program was utilized for the second PCR amplification: 95°C for 3 min followed by 8 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final elongation at 72°C for 5 min. A further clean up protocol using AMPure beads XP purification system (Beckman Coulter, UK) is performed. Amplicons were quantified using the Qubit® 2.0 Fluorometer (Invitrogen) and pooled equimolar to 4 nM. The pool was denatured with 0.2 M NaOH, further dilution with hybridization buffer to 20 pM and combined with denatured 30% PhiX. Samples were sequenced on the Illumina MiSeq platform at the Fundacion FISABIO (Valencia, Spain) facility using a 2x300 nucleotide paired reads protocol. Sequencing raw data were deposited at European Nucleotide Archive (ENA) and received the following ID: PRJEB14943.

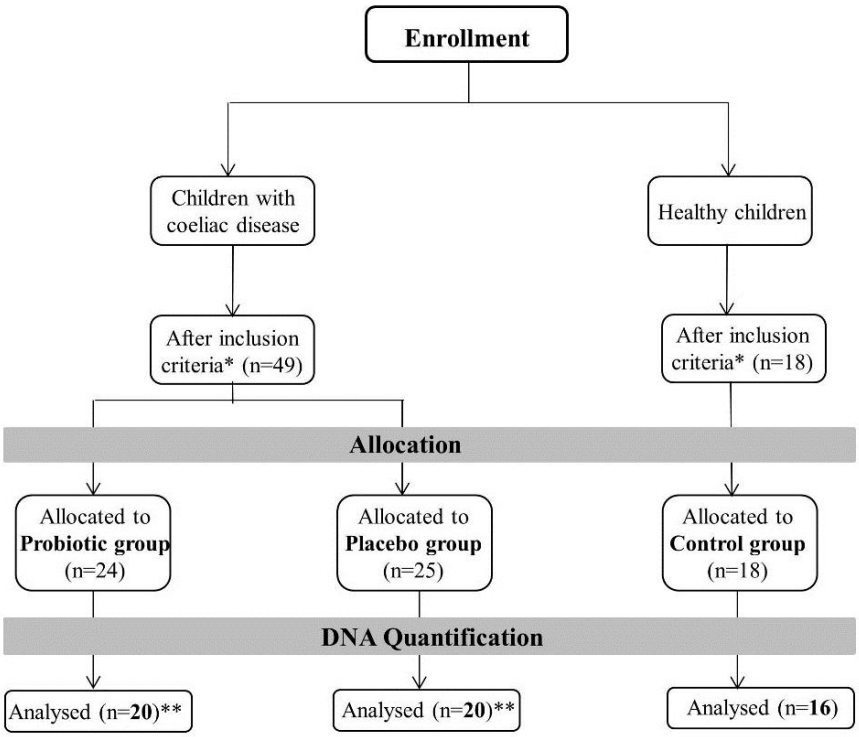

Figure 1: Summary of inclusion/exclusion criteria adopted for samples selection. Samples who showed the right DNA quantification level have been sequenced. *Inclusion criteria are summarized in Klemenak et al. [23]. **Analysis was performed at the beginning of the study (T0) and after 3 months of treatment (T1).

2.4 Bioinformatics and statistical analyses of NGS experiment

Several bioinformatics pipelines have been used to analyse the amount of data produced during this project. The first step of analysis was represented by quality controls of the generated raw data, which are essential to be confident of the quality of the experimental results. For this purpose, the FastQC 0.11.4 software (Babraham Bioinformatics) was used for a rapid visualization of sequences quality, then with the prinseq-lite.pl script sequences have been trimmed according to various quality criteria: first of all sequences with less than 50 bp were eliminated, then remaining reads were analysed with a sliding-window approach of 20 bp, within this range each sequence with a mean quality lower than 20 was removed [26].

After that, the fastq-join tool from the ea-tools suite [27] was used to join forward and reverse sequences. The last quality control step was represented by the elimination of chimeric sequences using the Usearch tool (http://drive5.com/usearch/). Once high-quality double-stranded reads were obtained, they were aligned to the 16S reference sequences database at the RDP database project to identify the microbial community with the RDP classifier tool [28]. RDP classifier outputs have been then processed through several R software packages, such as *vegan*, *reshape2*, *RDPutils* and *phyloseq* in order to estimate various biodiversity indexes and to perform the principle statistics analyses on taxonomic profiles. Finally, data have been normalized and the function exactTest() of the *edgeR* package was used to evaluate the effective microbial differentiation among the studied groups [29].

2.5 Absolute quantification of selected microbial groups using quantitative PCR (qPCR)

Quantification of selected microbial groups i.e. *Bidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis* group (comprising the species *B. fragilis, B. distasonis, B. ovatus, B. thetaiotaomicron, B. vulgatus*), *Clostridium sensu stricto* or cluster I and total enterobateria, was carried out with real-time PCR on DNA extracted from faecal samples. The assays were performed with a 20 µL PCR amplification mixture containing 10 µL of Fast SYBR® Green Master Mix (Applied Biosystems), optimized concentrations of primers (Table 1 and 2), H_2O molecular grade and 2 µL DNA extracted from faecal samples at a concentration of 2.5 ng/µL for all the assays. The primer concentrations were optimized through primer optimization matrices in a 48-well plate and evaluating the best Ct/ΔRn ratio. The different primers were also checked for their specificity using the database similarity search program nucleotide-nucleotide BLAST [30]. Moreover, to determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification. The data obtained from the amplification were then transformed to obtain the number of bacterial Log CFU/g faeces according to the rRNA copy number available at the rRNA copy number database [31]. Standard curves were constructed using 16S rRNA PCR product of type strains of each target microorganism. PCR products were purified with a commercial kit DNA purification system (NucleoSpin® Extract II kit, MACHEREY-NAGEL GmbH & Co. KG, Germany) and the concentration measured at 260 nm. Serial dilutions were performed and 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 copies of the gene per reaction and were used for calibration.
Data of microbial counts were subjected to T-test in order to evidence significant differences between treated and Control group of subjects.

Table 1. Primer sequences and qPCR conditions used in the different assays.

Target microorganisms	Primer sequences (5'-3')	Amplicon lengh (bp)	References	Annealing T
Bifidobacterium spp.				
BiTOT-F	TCGCGTCYGGTGTGAAAG	243	[32]	55 °C
BiTOT-R	CCACATCCAGCRTCCAC			
Lactobacillus spp.			[33]	60 °C
Lac-F	GCAGCAGTAGGGAATCTTCCA	349		
Lac-R	GCATTYCACCGCTACACATG			
B. fragilis group				
Bfra-F	CGGAGGATCCGAGCGTTA	92		
Bfra-R	CCGCAAACTTTCACAACTGACTTA		[34]	58 °C
Enterobacteria				
Eco 1457F	CATTGACGTTACCCGCAGAAG AAGC	195	[35]	63 °C
Eco 1652R	CTCTACGAGACTCAAGCTGC			
Clostridium cluster I				
CI-F1	TACCHRAGGAGGAAGCCAC	232	[36]	52 °C
CI-F2	GTTCTTCCTAATCTCTACGCAT			

Table 2. qPCR amplification protocols and primer concentrations

Target microrgansims	Initial denaturation	Denaturation	Annealing	Cycles	Fw nM	Rev nM
Bifidobacterium spp. BiTOT F/BiTOT-R	95 °C – 20 s	95 °C – 30 s	60 °C – 30 s	40	200	300
Lactobacillus spp. LAC-F/LAC-R	95 °C – 20 s	95 °C – 30 s	63.5 °C – 30 s	40	200	200
Bacteroides fragilis group Bfra-F/Bfra-R	95 °C – 20 s	95 °C – 30 s	60 °C – 30 s	40	300	300
Enterobacteria Eco-F/Eco-R	95 °C – 20 s	95 °C – 30 s	60 °C – 30 s	40	400	400
Clostridium cluster I CI-F1/CI-F2	95 °C – 20 s	95 °C – 30 s	60 °C – 30 s	40	200	200

3. Results

3.1 Metagenomic analysis

The V3-V4 region of 16S rDNA gene was sequenced from 96 DNA samples using the Illumina MiSeq platform. A total dataset of 4,348,432 filtered high-quality joined reads (excluding the undetermined sequences) was thus generated, about 46,259 sequences per sample, with a mean quality between 30 and 35. Two samples were excluded from the whole dataset because they did not pass the established quality threshold.

Massive sequencing revealed the presence of six phyla (5 belonging to Bacteria and 1 to Archaea) with a relative abundance higher than 1%, which were: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia* and *Euryarchaeota*. The obtained phyla had a different distribution among the five groups of examined subjects as highlighted in the heatmap (Figure 2), in particular in the *Firmicutes* and *Bacteroidetes* phyla.

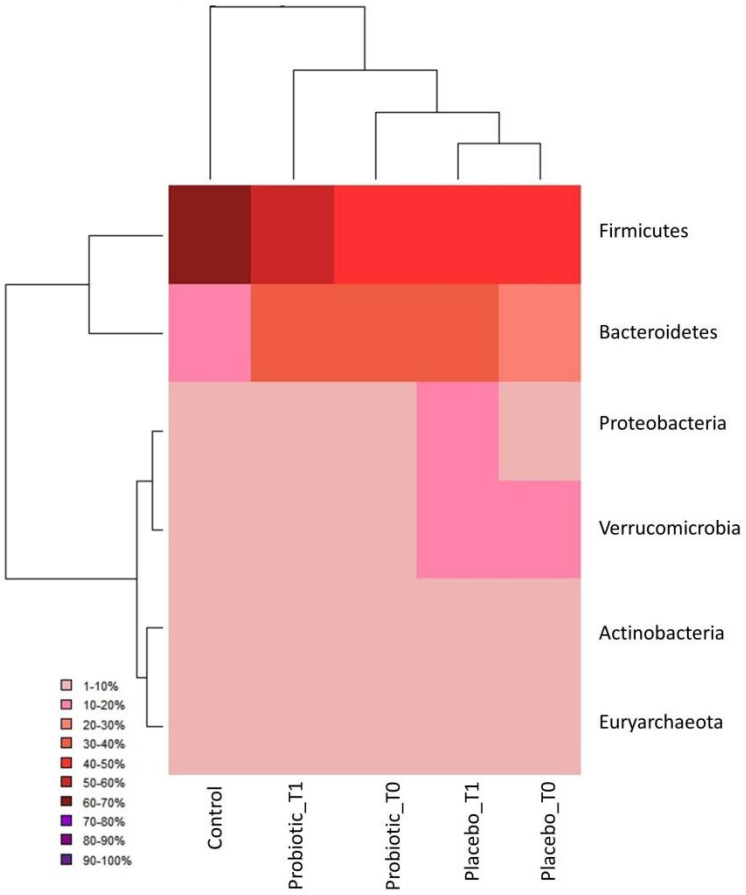

Figure 2: Hierarchically clustered heatmap: Sample groups are reported in column, while phyla are reported in row.

In particular, the *Firmicutes* phylum showed the highest representativeness in the Control group (accounting for 60-70% of the total microbial community), whereas it reached 50-60% in Probiotic T1 and 40-50% in the rest of CD patients (Probiotic T0, Placebo T0, Placebo T1 groups).
On the other hand, the *Bacteroidetes* phylum was more abundant within CD subjects (20-40 %) than in the Control group subjects (10-20%). The other phyla were more evenly distributed among groups, with the only difference for *Proteobacteria* and *Verrucomicrobia* that were more represented in the Placebo group (~10-20%). Moreover the hierarchical cluster analysis combined to the heatmap, pointed out that the Probiotic T1 group occupied an intermediate position between the Control group and the rest of CD individuals, being thus considered as an outlier with respect to the other disease clusters because of its closer relationship with control subjects.
From the comparison between the CD subjects and the Control group microbiota emerged a marked difference in the ratio of *Fimicutes/Bacteroidetes.* Figure 3 shows values of ratio *Fimicutes/Bacteroidetes* calculated for each group of subjects. CD subjects had a ratio values lower than the Control group thus meaning a high proportion of *Bacteroidetes* (Gram negative) with respect to *Firmicutes* (Gram positive). The administration of the probiotic for three months was found to increase the ratio value due to the higher level of *Firmicutes* phyla than *Bacteroidetes*.

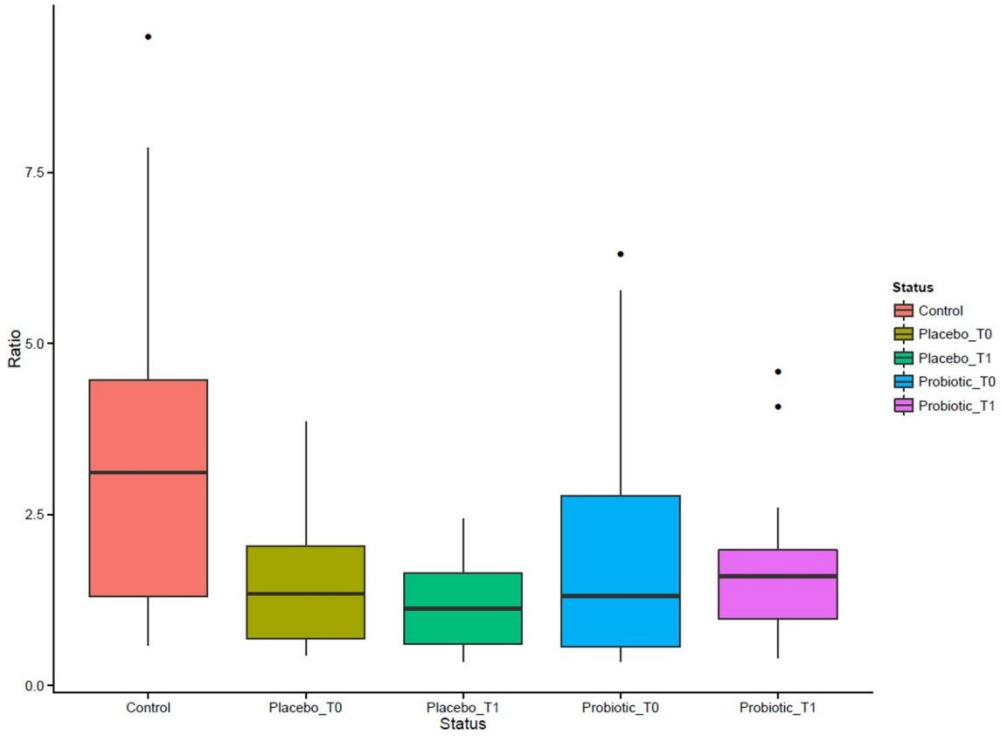

Figure 3: The *Firmicutes/Bacteroidetes* ratio

Following the data normalization procedure and assignation of statistical significance described in the material and method section, several comparisons between pair of groups were performed in order to identify which phyla could distinguish the microbiota of Control group from that of CD patients not assuming the probiotic formulation, and from Probiotic T1 (Figure 4).

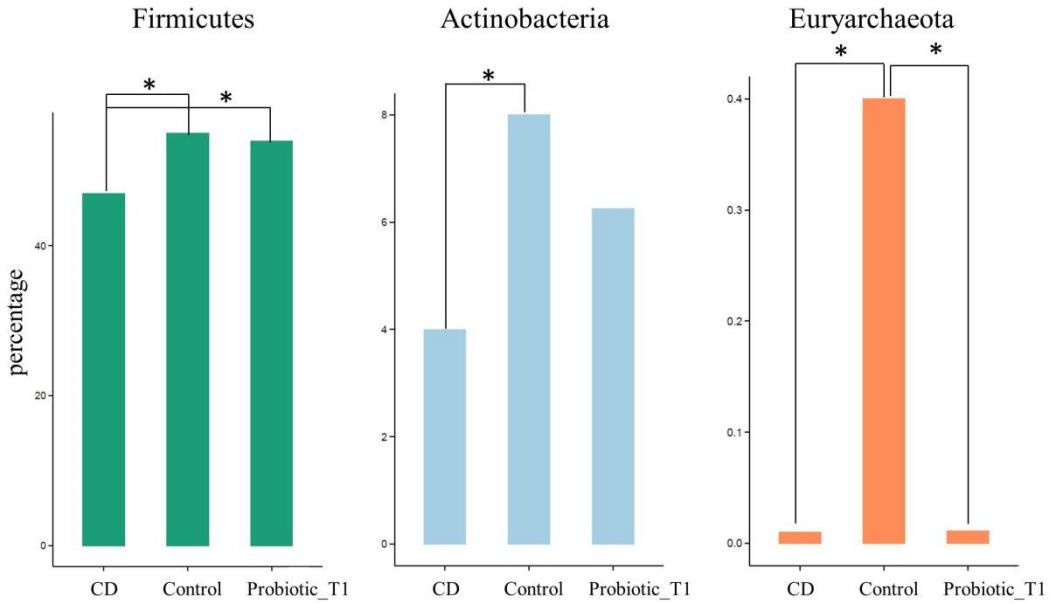

Figure 4: Relative abundance of the three phyla that show a statistical significance difference among CD, Control and Probiotic T1. CD group is composed by Probiotic T0 , Placebo T0 and Placebo T1 samples. The * indicates p<0.01. Supporting information on relative abundance and p-values in Table S2a-b.

Statistical analyses confirmed that *Firmicutes* were significantly lower in CD subjects not receiving the probiotic formulation compared to Controls and Probiotic group (p<0.01). Similar results were found for *Actinobacteria* that were underrepresented in the CD group and increased after the administration of bifidobacteria, although not reaching the abundance found in the controls. A further discernment regarded the *Euryarchaeota* phylum belonging to *Archaea* that was almost exclusively present in the Control group. The same analysis was repeated comparing the microbial composition of the Control group with the Probiotic groups before and after the probiotic administration (respectively Probiotic T0 and Probiotic T1) (Figure 5). The comparison highlighted an increase in the relative abundance of *Firmicutes* (p <0.01) and *Actinobacteria*, due to the effect of probiotic administration. On the other hand it was possible to observe a slight decrease of the abundance of *Proteobacteria* while the *Euryarchaeota* phylum kept unchanged after the treatment.
The same comparison was carried out at the family taxonomic level. Within the *Firmicutes* phylum, two families, which are poorly represented in the Probiotic T0 group, showed instead a higher level in both the Probiotic T1 and the Control groups: *Lactobacillaceae* and *Gracilibacteraceae*. In particular both bacterial families showed a significant different abundance between Probiotic T0 and Probiotic T1 groups, whereas no differences were observed between Probiotic T1 and Control groups. In contrast, Probiotic T1 subjects demonstrated a high percentage of unclassified *Deltaproteobacteria* families. Moreover this analysis enabled to identify the *Methanobacteriaceae* family as almost exclusively present within the Control group (Figure 6).

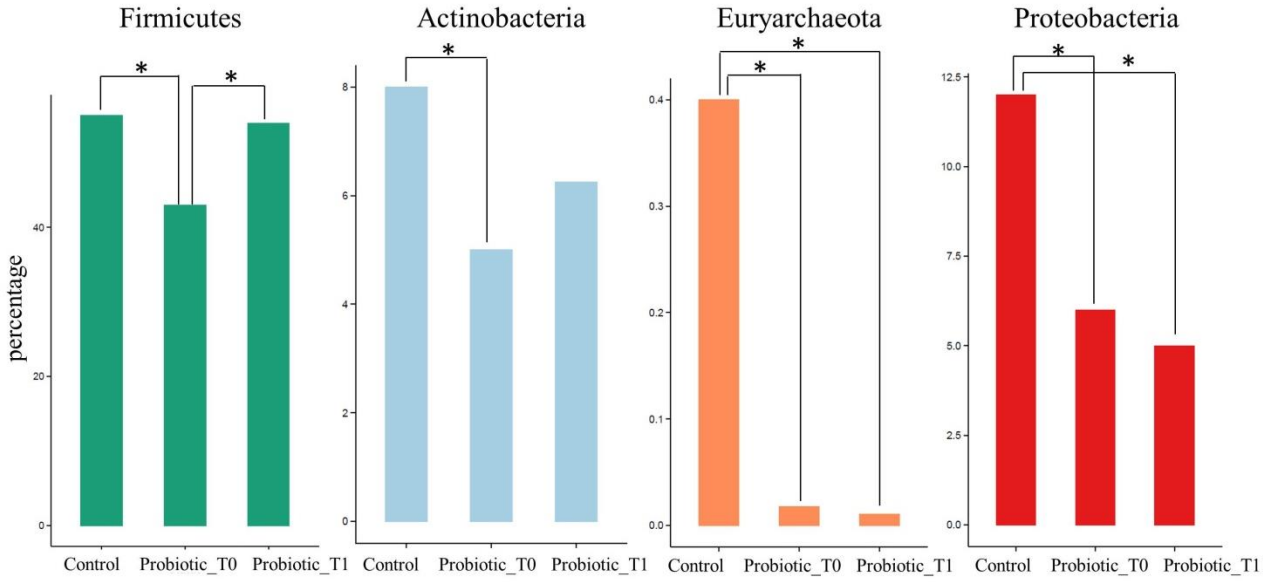

Figure 5: Significant differences in phyla relative abundance among Control, Probiotic T0 and Probiotic T1 groups. The * indicates p<0.01. Supporting information on relative abundance and p-values Table S2c-d.

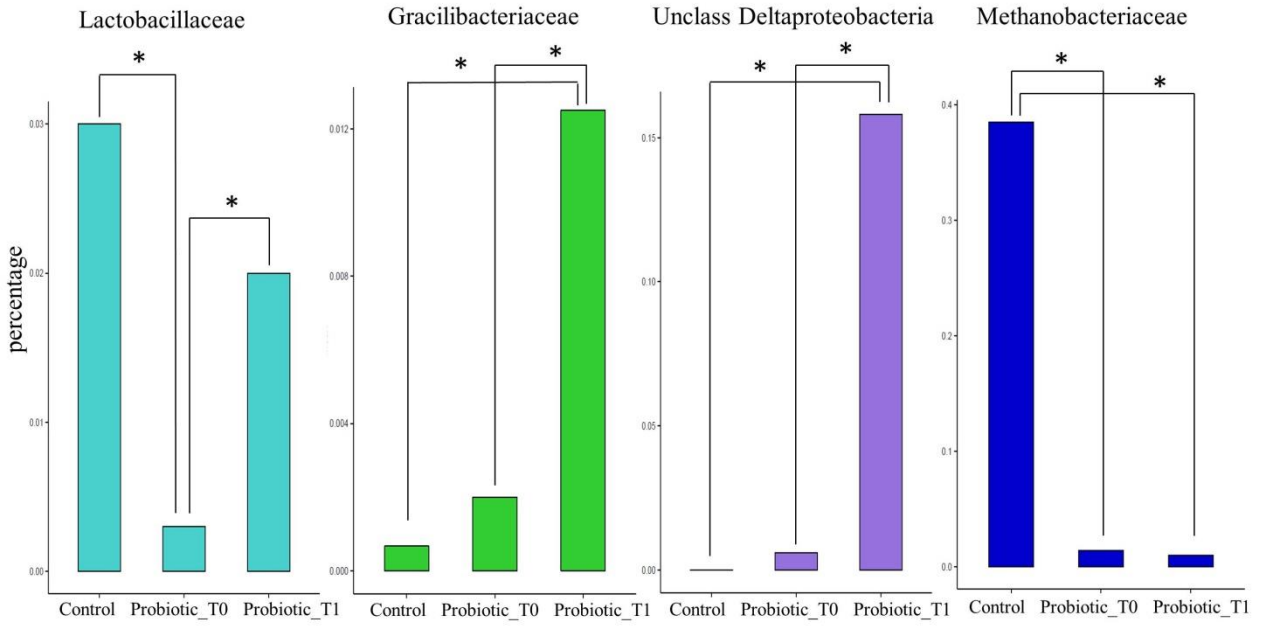

Figure 6: Statistical significant differences in families relative abundance among Control, Probiotic T0 and Probiotic T1 groups. The * indicates p<0.01. Supporting information on relative abundance and p-values in Table S2e-f.

The α-diversity indices (Observed, Chao1 and Shannon) were computed for all OTUs founded in the five groups of samples as reported in Figure 7. No significant changes in OTUs composition among the studied groups were observed. Particularly, the observed raw biodiversity, as well as the Chao1 index, were slightly higher in the control samples than in all the other groups, but the differences were not significant. Even the Shannon index indicated similar trends among all groups, with a mean value of about 3. This similarity among groups was further confirmed by the application of Wilcoxon test on these indices, which indicated the totally absence of significant differences.

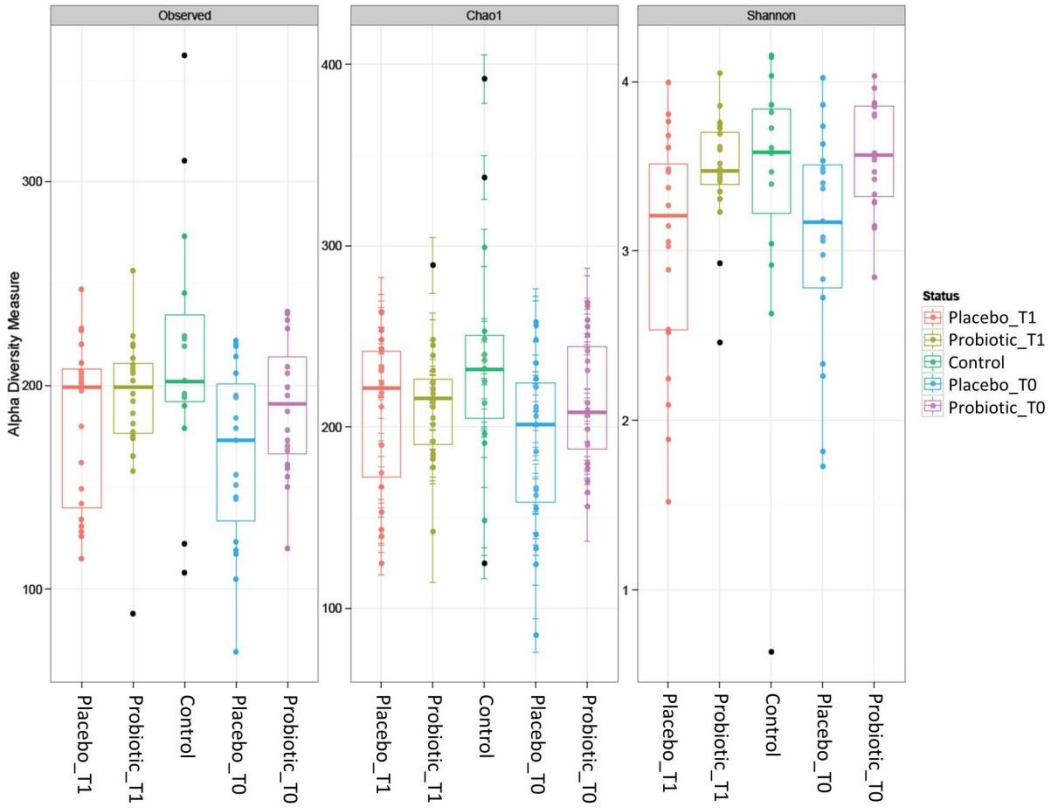

Figure 7: α-diversity indices among the studied groups.

3.2 Quantification of selected microbial groups in faecal samples

qPCR analysis was carried out in order to obtain the absolute quantification of selected microbial groups as a supplementary information able to complete the microbial profile of the examined subjects. Faecal samples were collected and DNA extracted at two sampling times for CD subjects, on enrolment (Probiotic T0 + Placebo T0) and at the end of the three months intervention with probiotic or placebo (T1), and once for healthy individuals (control group). Quantification regarded specific microbial genera typical of the human gut: *Bifidobacterium* spp., *Clostridium sensu stricto*, *Bacteroides fragilis* group (comprising the most abundant species in human, i.e. *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. vulgatus*), and larger microbial group: *Lactobacillus* group which include *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weisella* species, total enterobacteria comprehensives of a larger number of Gram negative intestinal bacteria. The average microbial counts obtained are shown in Table 3.

Quantification of *Bifidobobacterium* spp. evidenced a slightly higher value in the subjects affected by CD at T0 with respect to the control group, although this difference was not significant. The comparison between subjects belonging to the probiotic group before and after the treatment showed that the administration of the probiotic formula containing *Bifidobacterium breve* led to a slight increase of bifidobacteria counts from 7.64 ± 1.01 to 8.06 ± 0.98 Log CFU/g of faeces. *Lactobacillus* spp. group analysis revealed that healthy subjects (Control group) had a higher presence of members of this group compared to CD patients, which on the contrary, showed a great heterogeneity in the distribution (Figure 8). ANOVA test revealed that the difference was statistically significant ($P< 0.01$). The opposite trend was found for members of *Bacteroides fragilis* group showing a higher median in CD subjects compared to healthy subjects, as shown in the box plot (Figure 9). The box plot relative to healthy subjects is shorter than the other one and it also shows a higher median value but a narrower distribution of the data. ANOVA test revealed that the difference is statistically significant ($P < 0.01$). CD patients showed more than 8.70 Log CFU/g of faeces of *Bacteroides fragilis* group bacteria. No significant differences were recorded concerning changes in the levels of *Bacteroides* due to treatment with probiotics.

With regard to enterobacteria, they were more abundant in the control group compared to CD patients: 8.29 ±0.80 and 7.10 ±1.24 CFU/g respectively. This trend can also be outlined from the graphs reported in Figure 10 which clearly shows that the median value of control group is higher than CD groups, the latter showing a lower level of enterobacteria with a higher heterogeneity. Furthermore, after the three months of probiotic treatment it was possible to observe a decreased level of enterobacteria in Probiotic T1 (Figure 10 and Table 3). Regarding *Clostridium sensu stricto* its quantification was lower than the other microbial groups (values from 5.83 to 6.19 Log CFU/g of faeces). No statistical differences resulted from the comparison between control and CD patients and between Probiotic and Placebo groups.

Table 3 Mean counts of different microbial groups analyzed in stool samples expressed as Log CFU/g of faeces

Target	**Log no. CFU/g of faeces**				
	Probiotic group		**Placebo group**		**Control group**
	T0	**T1**	**T0**	**T1**	**T0**
Bifidobacterium spp	7.64±1.01	8.06±0.98	7.82±0.80	7.74±0.73	7.26±0.92
Lactobacillus spp	6.87±1.08	6.92±0.95	7.21±0.80	7.04±0.97	7.84±0.58
B. fragilis group	8.73±0.79	8.71±0.77	8.74±0.76	8.84±1.03	7.46±1.47
Enterobacteria	7.10±1.24	6.75±1.29	7.25±1.81	7.63±1.48	8.29±0.80
Clostridium sensu stricto	5.97±0.96	5.83±0.87	6.17±0.95	6.19±0.81	5.86±0.80

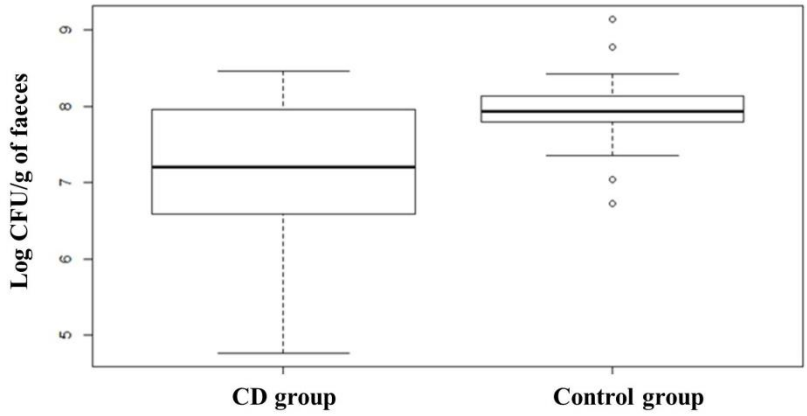

Figure 8: Box plots showing qPCR analysis of *Lactobacillus* group expressed in log CFU per gram of faecal sample relative to CD group and Control group. CD group is composed by Probiotic T0 , Placebo T0 and Placebo T1 samples. Statistical difference between the two groups (p-values of < 0.01)

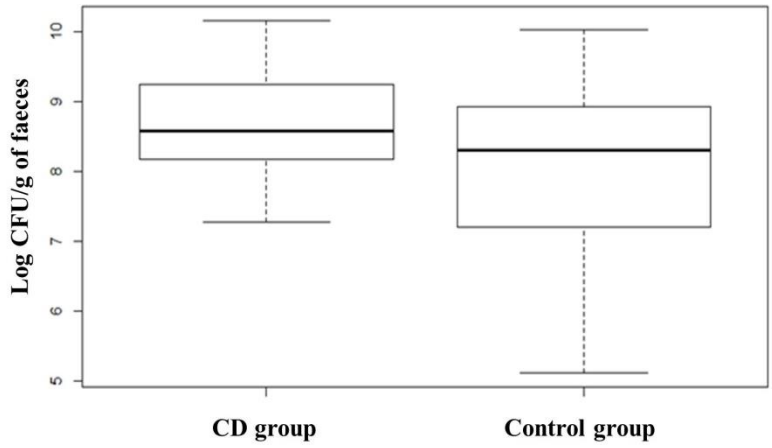

Figure 9: Box plots showing qPCR analysis of *Bacteroides fragilis* group expressed in log CFU per gram of faecal sample relative to CD group and Control group. CD group is composed by Probiotic T0 , Placebo T0 and Placebo T1 samples. Statistical difference between the two groups (p-values of < 0.01).

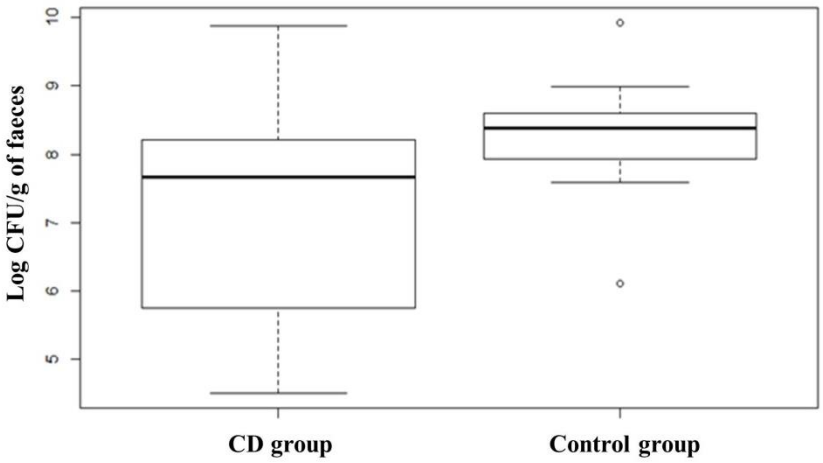

Figure 10: Box plots showing qPCR analysis of total enterobacteria expressed in log CFU per gram of faecal sample relative to CD group and Control group. CD group is composed by Probiotic T0 , Placebo T0 and Placebo T1 samples. Statistical difference between the two groups (p-values of < 0.01).

4. Discussion

This work was focused on the characterization of the major changes occurring in the intestinal microbiota of CD patients on a GFD and on the evaluation of the effects that the administration of two *B. breve* strains (B632 and BR03) may have on these patients.

In the last few years a particular attention has been paid on the correlation between gut microbiota composition and CD. Several studies demonstrated an increase in Gram negative bacteria, mainly belonging to the *Bacteroidetes* phylum, at the expense of microorganisms of the *Actinobatceria* and *Firmicutes* phyla in subjects with active disease [7,8,10], in agreement with the results registered in other chronic inflammatory gastrointestinal diseases such as inflammatory bowel disease [37]. However these differences did not allow to identify a coeliac microbiota signature directly linked to CD [6].

Although data regarding the health promoting properties of bifidobacteria and more in general of probiotic microorganisms are well documented, their role in the treatment of CD has been scarcely investigated. The two strains administered in this work, *B. breve* B632 and BR03, are known to possess anti-inflammatory activity stimulating intestinal cells *in vitro* to produce IL-6 and IL 10, respectively [38,39] and have been previously characterized for safety issues such as the absence of transmissible antibiotic resistance traits and toxicity towards gut epithelial cells. In addition, the two strains in combination showed a great capability of colonizing the gut of healthy children [40]. In relation to CD, a preliminary important outcome obtained from the administration of the described probiotic formulation to CD patients was the reduction of pro-inflammatory cytokine TNF-α in blood samples of CD subjects on a GFD after three months of treatment, as reported in Klemenak et al. [23].

The first interesting evidence that emerges from the present study is the absence of a severe intestinal dysbiosis in CD patients on a GFD diet, as shown by the comparison of the α-diversity similarity indices and the absence of statistically significant differences in OTU variability in the

analysed cohort of CD patients with respect to the Control group. On the contrary, literature data related to active disease patients non-adherent to a GFD showed the presence of extensive changes in the microbial composition [8]. Therefore, the strict adherence to the GFD partially recovers the intestinal equilibrium status.

However, the results obtained in this study showed a significant quantitative difference in some microbial groups by qPCR and by metagenomic analysis in CD patients with respect to the Control group. The elaboration of the microbial relative abundance data obtained by Illumina MiSeq sequencing were able to clearly separate CD subjects from the Control group ones. The lower number of *Bacteroidetes* phylum in CD patients with respect to the Control group was supported by *B. fragilis* group quantification by qPCR and it is consistent with the results of another study on CD patients on GFD [7]. The obtained results are also in agreement with the observation that CD subjects present an imbalance in the *Firmicutes*/*Bacteroidetes* ratio, usually lower in CD patients, and this ratio is not completely restored in patients under a GFD [41]. Moreover the probiotic administration induced an evident increase of *Firmicutes* abundance while maintaining a similar percentage of *Bacteroidetes*, thus resulting in a higher value of the *Firmicutes*/*Bacteroidetes* ratio. In addition, the Control group microbiota seems to be characterized by a higher percentage of *Actinobacteria* and *Euryarchaeota*. The association between CD disease status and a lower presence of *Actinobacteria* has already been described [42]. Particularly interesting although not yet described in the literature is the result regarding the *Euryarchaeota* phylum, which is highly represented in the Control group, but almost absent in the coeliac subjects. The same applies for the *Methanobacteriaceae* family. This evidence could conceivably be linked to differences in the dietary habits of the two groups of subjects. Recent research works focused on *Euryarchaeota* highlighted their ability to promote polysaccharide degradation and absorption of fatty acids, thus they seem to play a role in energy extraction from degradation of organic compounds [43]. Grain is the most common source of polysaccharides in modern human populations, thus the important reduction of archaea microorganisms within coeliac group on GFD is linked to their different nutritional status, in particular to the compliance of the GFD and the consequent lower polysaccharide intake.

Focusing on the effects of the administration of the *B. breve* strains on microbial composition, an increase of members of the *Actinobacteria* phylum (NGS) and bifidobacteria (qPCR) have been detected in the CD subjects after three months of probiotic supplementation, although the increase was not statistically significant. One of the possible reason could be the short duration of the treatment, furthermore it is already known that, after the weaning period, the microbiota is resilient to changes [44]. The treatment with the *B. breve* strains has therefore not caused major changes at the level of the genus or phylum to which the probiotic belongs, as it might have been expected, but the intake of the probiotic has nevertheless acted as a "trigger" element for the increase of *Firmicutes* and the restoration of the physiological *Firmicutes/Bacteroidetes* ratio. By reaching the microbial family level of analysis, it was possible to get more details on the effect of probiotic administration, allowing to reach the conclusion that two *Firmicutes* families (*Lactobacillaceae* and *Gracilibacteraceae*) changed their relative abundances upon probiotic treatment (Probiotic T1 group), particularly *Lactobacillaceae* that reached almost the values that characterized the Control group. Other studies have also observed a lower presence of *Lactobacillaceae* in CD patients, indicating a close relationship between this pathological condition and the bacterial family [9]. This means that the probiotic has restored the normal amount of *Lactobacillaceae* members belonging to

these families within the treated individuals. It remains to be explained why the administration of such a *Bifidobacterium* strain have affected *Lactobacillaceae* species. This could be related to a high ability of *Bifidobacterium* to deep influence gut microflora composition, by enhancing the blooming of some species and antagonizing others probably by the effect of the production of metabolites such as acetic acid [45]. In particular, there are evidences that *Bifidobacterium* support *Lactobacillaceae* development [46]. Moreover it is highly probable that the decrease of TNF-α observed within treated individuals, is closely linked to the increase of lactobacilli, with their anti-inflammatory function promoted by the administration of *Bifidobacterium* [47,48].

In conclusion, the present study demonstrated that three months administration of *B.breve* strains could make the intestinal microbiota of coeliac patients more similar to that of healthy individuals, restoring the abundance of some microbial communities that characterize the typical physiological condition.

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Supplementary Materials

Table S1. Description of the study groups at the begin of intervention

	Placebo group	Probiotic group	Control group
No. patiens	20	20	16
Sex (M/F)	8/12	6/14	7/9
Age (year)	10.53 ± 3.87	10.23 ± 3.18	8.80 ± 5.12
Time on GFD (year)	7.16 ± 4.98	5.43 ± 3.40	-
Compliance to GDF	80 %	90 %	-

Table S2. *p*-values among groups

domain	phylum	CD - Control	CD - Probiotic T1	Control - Probiotic T1
Archaea	Euryarchaeota	0.000079	n.s.	0.00009
Bacteria	Actinobacteria	0.0036	n.s.	n.s.
Bacteria	Firmicutes	0.0022	0.0022	n.s.

Table S3. Mean values of relative abundance with standard deviation.

Phylum	CD	Control	Probiotic T1
Euryarchaeota	0.01±0.003	0.4±0.5	0.011±0.02
Actinobacteria	4±3	8±5	6.25±4
Firmicutes	47±15	55±17	54±18

Table S4. *p*-values among groups

Domain	Phylum	Control – Probiotic T0	Control – Probiotic T1	Probiotic T0 – Probiotic T1
Archaea	Euryarchaeota	0.0000195	0.00009	n.s.
Bacteria	Actinobacteria	0.0021	n.s.	n.s.
Bacteria	Firmicutes	0.0003926	n.s.	0.000536
Bacteria	Proteobacteria	0.0031	0.0018	n.s.

Table S5. Mean values of relative abundance with standard deviation

Phylum	Control	Probiotic T0	Probiotic T1
Euryarchaeota	0.4±0.5	0.018±0.03	0.011±0.02
Actinobacteria	8±5	4.8±3	6.25±4
Firmicutes	55±17	48.2±17	54±18
Proteobacteria	12±6	6.23±5	5.134±4.8

Table S6. *p*-values among groups

Domain	Phylum	Control - Probiotic T0	Control - Probiotic T1	Probiotic T0- Probiotic T1
Archaea	Euryarchaeota	0.0000195	0.00009	n.s.
Bacteria	Actinobacteria	0.0021	n.s.	n.s.
Bacteria	Firmicutes	0.0003926	n.s.	0.000536
Bacteria	Proteobacteria	0.0031	0.0018	n.s.

Table S7. Mean values of relative abundance with standard deviation

family	Control	Probiotic T0	Probiotic T1
Unclass Deltaproteobacteria	0.0001±0.00065	0.02±0.005	0.152±0.0039
Lactobacillaceae	0.028±0.0035	0.005±0.00065	0.019±0.006
Gracilibacteraceae	0.0008±0.00005	0.003±0.00022	0.0123±0.0048
Methanobacteriaceae	0.363±0.1	0.01±0.002	0.0095±0.0008

Paper 4

Clinical intervention using *Bifidobacterium* strains in coeliac disease children reveals novel microbial modulators of TNF-α and short-chain fatty acids

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Abstract

Background & Aims: Coeliac disease (CD) is an immune-mediated systemic disease, caused by ingestion of gluten in genetically predisposed individuals. Gut microbiota dysbiosis might play a significant role in pathogenesis of chronic enteropathies and its modulation can be used as an intervention strategy in CD as well. In this study, we aimed to identify correlations between faecal microbiota, serum tumor necrosis factor α (TNF-α) and faecal short-chain fatty acids (SCFAs) in healthy children and children with CD after administration of probiotic *Bifidobacterium breve* BR03 and B632.

Methods: A double-blind placebo-controlled study enrolled 40 children with CD (CD) and 16 healthy children (HC). CD children were randomly allocated into two groups, of which 20 belonged to the placebo (PL) group and 20 to the Probiotic (PR) group. The PR group received a probiotic formulation containing a mixture of 2 strains, *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) in 1:1 ratio for 3 months. Subsequently, for statistical analysis, blood and faecal samples from CD children (on enrolment - T0 and after 3 months, at the end of intervention with probiotic / placebo - T1) and HC children were used. The HC group was sampled only once (T0).

Results: *Verrucomicrobia*, *Parcubacteria* and some yet unknown phyla of *Bacteria* and *Archaea* may be involved in the disease, indicated by a strong correlation to TNF-α. Likewise, *Proteobacteria* strongly correlated with faecal SCFAs concentration. The effect of probiotic administration has disclosed a negative correlation between *Verrucomicrobia*, some unknown phyla of *Bacteria*, *Synergistetes*, *Euryarchaeota* and some SCFAs, turning them into an important target in microbiome restoration process. *Synergistetes* and *Euryarcheota* may have a role in the anti-inflammatory process in healthy human gut.

Conclusions: Our results highlight new phyla, which may have an important relation to disease-related parameters, CD itself and health.

***Keywords**:* coeliac disease; children; *Bifidobacterium breve*; intestinal microbiota; TNF-α; short-chain fatty acids.

Introduction

Diagnostic rates of coeliac disease (CD) are rising, leading towards an estimated global prevalence of about 1% [1]. Ingestion of wheat and other gluten-containing cereals causes a specific damage to the small intestinal mucosa, a typical pathology of CD, which is considered an autoimmune enteropathy [2].

The role of susceptibility genes in the pathogenesis of CD has been described [3,4]. However, additional environmental factors are involved, as only 2-5 % of CD-related gene carriers eventually develop the disease [5]. Epidemiological and clinical data suggest a role of various environmental factors in the pathogenesis of CD, such as infections, early feeding practices [6], antibiotic administration, mode of delivery and breastfeeding [7]. In addition, alteration of gut microbiota may also play an important role in the disease development. Whether it is a cause or a consequence of the disease, remains unclear [8–10]. Due to inconsistent findings concerning both active and non-active disease phase [11–15], CD still lacks a distinctive ′microbial footprint′, although some bacterial species may associate with the disease [16]. Moreover, microbial metabolites such as short-chain fatty acids (SCFAs) play an important role in trigger-response relationship between host diet, microbiota and homeostasis in many pathological conditions, also in CD [17,18]. However, changes in the faecal SCFAs pattern are a reflection of complex mechanisms [19] and studies about their relationship and effects on CD are scarce [20].

Application of probiotics in clinical practice has been frequently used due to their immunomodulatory [21,22] and microbiota modulation effects [23–26], demonstrated in several inflammatory and autoimmune diseases. Moreover, the effect of *Bifidobacterium* strains on gut microbiota composition and their applications as probiotics in infants have been reviewed [27] and their administration in one *in-vivo* study [25] has revealed an impact on *Firmicutes* abundance, resulting in an increase of *Firmicutes / Bacteroidetes* ratio. Furthermore, its immunomodulatory characteristics have been described *in-vitro* [28] and *in-vivo* [29–32]. Several *in-vitro* studies have shown that *Bifidobacterium* strains decrease levels of pro-inflammatory cytokines, such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin 2 (IL-2) [33–36]. Moreover, a decrease in TNF-α level after administration of *Bifidobacterium* strains has been reported *in-vivo* [30,31]. In fact, TNF-α secretion, triggered by an increased production of IFN-γ in CD, plays an essential role in inducing damage and inflammation of intestinal mucosa [26,37].

Our aim was to study the effects of *Bifidobacterium breve* BR03 and B632 administration on children with CD and to determine statistically significant correlations between faecal microbiota composition analysed by next generation sequencing, serum TNF-α and faecal SCFAs levels. To our knowledge, this is the first study on correlations between these parameters with the aim to evaluate their potential significance in CD pathogenesis.

Material and methods

Study design and sample collection

The research study was a double-blind placebo-controlled intervention involving 40 children with CD (CD) and 16 healthy children (HC), who were enrolled at the Department of Paediatrics, University Clinical Centre Maribor in a period from October 2013 to June 2014. The research was registered at https://www.clinicaltrials.gov (registration number: NCT02244047).

A selection of HC as control group was based on a clinical examination, excluding any clinical disorder or any acute and chronic illness status. None of HC was on medication or antibiotic therapy for at least one month preceding the research study. HC were children, matching on age and gender and consuming a regular (gluten containing) diet.

All invited CD children, aged from 1 till 19 years, were previously diagnosed with positive serologic markers for CD and had positive small bowel biopsy. Their CD diagnosis were established on ESPGHAN criteria for CD [38,39]. The children were consuming gluten-free diet (GFD) (different time periods - half a year to 15 years). Children with acute or chronic illness and children on permanent medication or antibiotics for at least one month preceding the research study were excluded. CD children were randomly allocated into two groups, of which 20 belonged to the placebo (PL) group and 20 to the Probiotic (PR) group. The PR group received a probiotic formulation containing a mixture of 2 strains, *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) in 1:1 ratio for 3 months. Probiotic and placebo packages contained 2 g of probiotic culture or placebo in a powder form. A daily dosage of each probiotic strain was 10^9 Colony Forming Unit (CFU) / g of powder. In both groups, cytokine analysis, analysis for CD serological markers (EMA, tTG) and clinical examination were performed (on enrolment (T0), at the end of intervention with probiotic / placebo (T1) and on follow up – 3 months after intervention period (T2)). A more detailed information about probiotic administration and inclusion / exclusion criteria of participating children has been described before [30].

Blood samples of CD children were collected 3 times (at T0, T1 and T2). The HC group was sampled only once (T0). Please refer to the article of Klemenak *et al*. [30] for more details. However, for statistical analysis, samples from periods T0 and T1 were collected (see section *Statistical analysis of NGS, SCFAs and TNF-α results*).

Faecal sample of CD children were collected twice, on T0 and T1. The HC group was sampled only once (T0). Please refer to the detailed description of faecal collection in Primec *et al.* [20].

Researchers carrying out DNA extraction, molecular (NGS) and HPLC analysis of faecal samples were blind to the children group identity (HC, PR and PL).

DNA extraction

DNA extraction from 200 mg of faeces, which was preserved at -80 °C, was accomplished with the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to manufacturer's instructions. A slight modification was performed, in order to improve the bacterial cell rupture [40]. A detailed protocol was described in Quagliariello *et al*. [25].

Preparation of DNA Libraries for Next-Generation Sequencing (NGS; Illumina MiSeq Sequencing)

Samples of the following 5 groups of children were subjected to sequencing: 20 PR group T0 and 20 PR group T1, 20 PL group T0 and 20 PL group T1 and 16 HC group T0. Libraries were prepared for amplification of V3-V4 region of the 16S rRNA gene, using forward and reverse primers [41], respectively: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′, and 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′. Their approximate length was 460 bp. A detailed NGS protocol was described in Quagliariello *et al*. [25].

SCFA analysis

SCFAs were derivatized and analysed by reverse-phase HPLC. Acetic, propionic and butyric acid were quantified and results were expressed in µmol / g of wet weight faeces. For derivatization procedure and HPLC analysis of SCFAs please refer to the article of Primec *et al.* [20].

TNF-α detection

After centrifugation, serum samples for TNF-α detection were collected and stored at -80 °C until analysis. Quantification was performed using a solid-phase enzyme-labeled chemiluminescent immunometric assay, according to the manufacturer's instructions (Immulite One, Siemens Healthcare Diagnostics). A more detailed information about the blood sampling procedure and further TNF-α detection is described in the article of Klemenak *et al*. [30].

Bioinformatics and statistical analyses of NGS experiment

In bioinformatics and statistical analyses, the generated raw data has been checked for quality levels, length and elimination of chimeric sequences in order to obtain reliable double-stranded reads for the 16S reference sequence database alignment at the Ribosomal Data Project (RDP). Finally, RDP outputs were processed and further statistically analyzed [25,42–44].

Statistical analysis of NGS, SCFAs and TNF-α results

Patients were grouped according to the treatment and disease, i.e. probiotic group (PR; PR group T0, PR group T1), placebo group (PL; PL group T0, PL group T1), healthy controls (HC group T0) and CD patients (CD group T0). The CD group T0 consisted of all CD patients at T0 (PR and PL group, both T0). Results from SCFAs (i.e. acetic, propionic, butyric acid and total SCFAs), microbial phylum abundance and TNF-α analysis from 6 groups were statistically correlated for placebo and probiotic groups at the beginning and at the end of probiotic intervention.
Obtained data were analysed using IBM SPSS Statistics 22.0 software (IBM Inc., Armonk, New York). Age differences between study groups were analysed using non-parametric Kruskal-Wallis H test. Correlations between two continuous variables were determined using non-parametrical Spearman correlation after Shapiro-Wilk test of data distribution normality. Where indicated, p value of ≤ 0.05 or ≤ 0.01 was considered statistically significant.

Results and discussion

NGS analysis

DNA was extracted out of 96 faecal samples and was sequenced using the Illumina MiSeq apparatus. Sequencing runs generated 4,348,432 joint reads with high quality pass filter with average of 46,259 sequence reads per sample with quality scores between 30 and 35. Two samples were excluded from further analysis due to low quality reads [25]. A detailed microbial profile of each group is shown in Supplementary Tables 1a – f.

Characteristics of study groups used for statistical analysis of correlations

Basic characteristics of children whose parameters were used for statistical analysis of correlations are summarized in Table 1. Three samples were excluded due to low quality reads in NGS and insufficient data.

Table 1. Cohort used for the correlation assessment.

	PR group (n = 20)	**PL group** (n = 19)	**HC group** (n = 14)	***P* value**
Age,Years	9.15 ± 4.35	10.53 ± 5.05	10.14 ± 6.01	0.709
Sex, M/F	4/16	6/13	5/9	/

Statistical analysis of correlations

1. Correlation values at T0 in CD patients

The CD patient group was analysed at the enrolment day (T0). Fig. 1 shows the results of statistically significant correlation values. TNF-α had a positive correlation to *Verrucomicrobia* (ρ = 0.404, p = 0.013) and a negative one to *Parcubacteria* (ρ = 0.396, p = 0.015). Moreover, a strong positive association and a high statistical significance (ρ = 0.532, p = 0.001) between TNF-α and unclassified *Bacteria* group and a positive correlation (ρ = 0.396, p = 0.003) between TNF-α and unclassified *Archaea* group was found, indicating that *Verrucomicrobia* and some yet unknown phyla, belonging to *Bacteria* and *Archaea*, may be involved in an increased production of TNF-α in CD patients, while *Parcubacteria* indicated a negative association with TNF-α. *Verrucomicrobi*a is commonly encountered in the colonic microbiota [7], but is relatively less than 10% abundant [45]. *Parcubacteria* is a largely unknown phylum, with representatives found in anoxic environments [46]. Indicated correlations could play an important role in the pathogenesis of the disease.
Proteobacteria correlated positively with acetic and propionic acid (ρ = 0.452, p = 0.004 and ρ = 0.331, p = 0.045, respectively), which resulted in a positive correlation between *Proteobacteria* and total SCFAs (ρ = 0.380, p = 0.017). *Proteobacteria* is the major gut-resident phylum of Gram negative bacteria and includes a wide variety of pathogens, including members of *Enterobacteriaceae* family. Furthermore, the phyla has been found characteristically increased in duodenal and faecal microbiota of CD patients [16,26,47–50]. Increased values of acetic, propionic

acid and total SCFAs in CD have been suggested before [20,51–53], describing them as a consequence of microbiota dysbiosis in the disease. Tjellström *et al.* [53] even described acetic acid as a potential pro-inflammatory agent. Positive correlation data obtained in our studies indeed indicate that *Proteobacteria* may be responsible for an increased acetic and propionic acid production in CD. *Proteobacteria*, *Bacteroides-Prevotella* group (*Bacteroidetes*) and *Bifidobacterium* spp. have been already described as acetate and propionate producers [54]. In contrast, butyric acid has been proposed to originate mostly from the metabolism of *Firmicutes* [18]. From the *Archaea* group, *Euryarchaeota* phylum also had a positive correlation (ρ = 0.351, p = 0.029) to acetic acid. *Euryarchaeota* phylum is the most commonly found *Archaea* in the human ecosystem, contributing to less than 10% of the total microbiota population [55]. *Euryarcheota* is known to metabolize nutrients and other microbial metabolites to end products such as acetate. This results in an increase of total SCFAs concentration and energy harvesting [56]. Till now, the potential relationship between *Euryarcheota* and acetic acid has not been linked to CD.

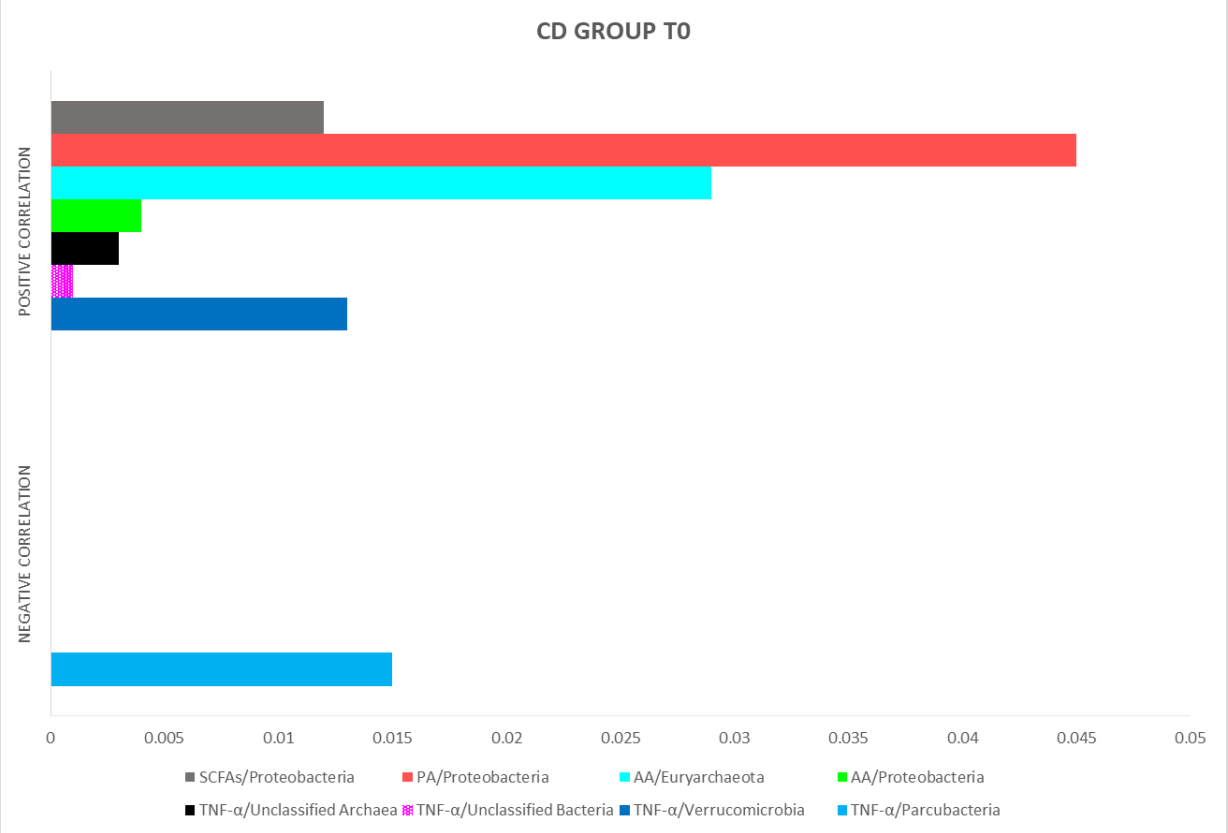

Fig. 1. Statistically significant correlations in the CD group at T0. CD: coeliac disease; T0: enrolment day; AA: acetic acid; PA: propionic acid; SCFAs: short-chain fatty acids; TNF-α: tumor necrosis factor alpha.

2. Correlation values at T0 in healthy children (HC)

Fig. 2 shows the results of statistically significant correlation values in the HC group at T0. Members of the *Firmicutes* phylum are mostly Gram positive bacteria, an abundant group comprising 80% of the intestinal microbiota [45] in healthy subjects and tend to decrease in number in CD patients [16]. Not surprisingly, proinflammatory TNF-α had a strong negative association and a high statistical significance (ρ = 0.660, p = 0.010) to *Firmicutes* and a negative correlation to *Euryarchaeota* (ρ = 0.654, p = 0.011). Apparently, the later relationship may play an important role only in the HC population, as it was not identified in the CD group T0. *Synergistetes* is evidently a minority phylum in human faeces with an abundance of 0.01% [57]. Regardless of the low quantity, the phylum appears to be relevant for human health [58] and its negative correlation (ρ = 0.658, p = 0.011) to TNF-α may indicate an important anti-inflammatory factor in healthy population.
Furthermore, acetic acid had a positive correlation (ρ = 0.569, p = 0.034) to *Candidatus Saccharibacteria*, a group of *Bacteria* still under investigation for its potential role in human health [59]. *Lentisphaerae* negatively correlated (ρ = 0.556, p = 0.039 and ρ = 0.584, p = 0.028, respectively) with butyric acid and total SCFAs. This phylum of *Bacteria* is closely related to *Verrucomicrobia*, but its activity and role in host microbiota still needs to be determined [58].

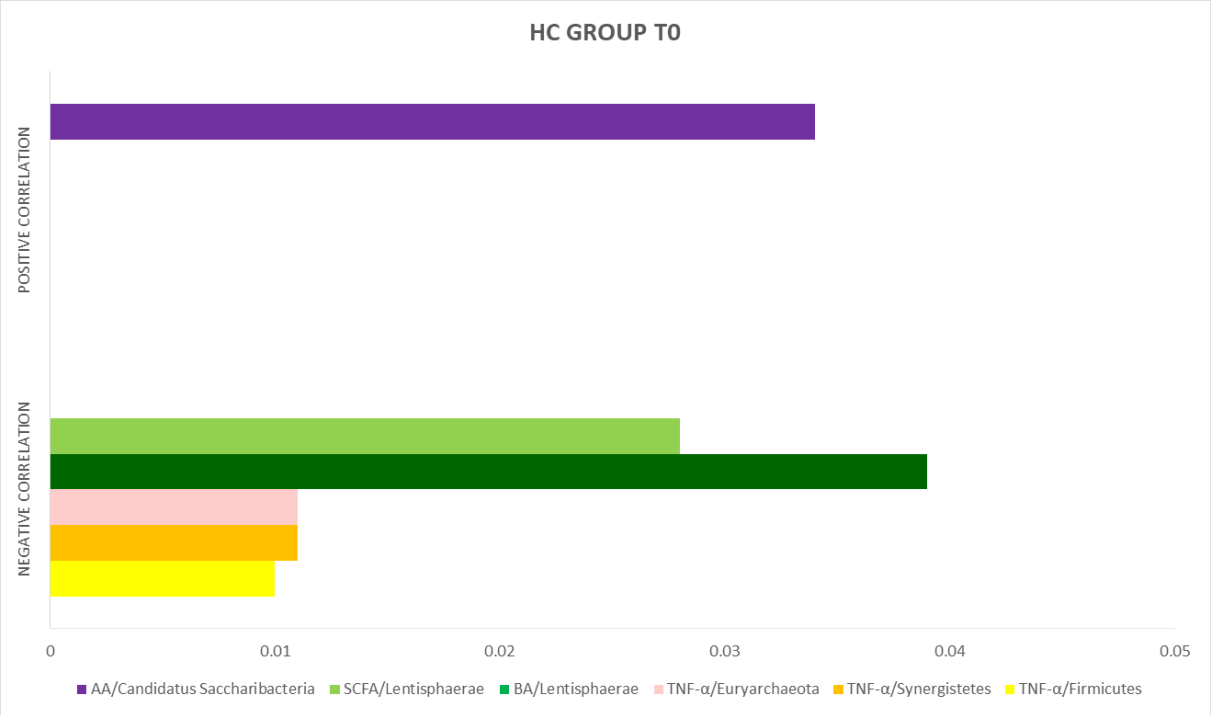

Fig. 2. Statistically significant correlations in the HC group at T0. HC: healthy children; T0: enrolment day; AA: acetic acid; BA: butyric acid; SCFAs: short-chain fatty acids; TNF-α: tumour necrosis factor alpha.

3. Effect of probiotics on correlation values in the PR group at T1

Significant correlations after probiotic administration are presented in Fig. 3. No significant correlations have been found in the PR group on the enrolment day (T0). However, several significant correlations emerged in the same group after 3-month treatment with the probiotic (PR group T1).

TNF-α is so far known for its important role in pro-inflammatory conditions. In fact, its appearance as an inflammatory mediator in CD patients have been already described [37,60]. However, in the work of Klemenak et al. (2015), the administration of both probiotic strains (*B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706)) revealed a decrease in TNF-α in PR group after 3 months compared to PL group. The baseline TNF-α levels in both groups were similar to the ones in HC group. As both groups (placebo and probiotic, respectively) had a compliance to GFD of 81% and 91%, the researchers concluded that the reduction in TNF-α occurred because of the combination of *B. breve* strains and GFD. Moreover, Quagliariello et al. (2016) reported that the 3-months probiotic administration in PR group affected the abundance of *Firmicutes phylum* by increasing their percentage, while keeping similar percentage of *Bacteroidetes*, thus resulting in an increase of *Firmicutes / Bacteroidetes* ratio. The ratio in CD subjects is normally lower, usually because of lower percentage of *Firmicutes* or higher percentage of *Bacteroidetes.* In relation to both parameters, an interesting observation has been found by evaluating the correlation results between TNF-α and *phylum Firmicutes*. After 3 months of probiotic administration, TNF-α had a negative correlation (ρ = 0.468, p = 0.038) to *Firmicutes*, which is in concordance with the article of Klemenak et al. (2015) and Quagliariello et al. (2016), revealing a decrease in TNF-α and re-establishment of the *Firmicutes / Bacteroidetes* ratio, respectively, upon probiotic treatment. Acetic acid correlated negatively (ρ = 0.502, p = 0.024; ρ = 0.498, p = 0.026 and ρ = 524, p = 0.018) with *Verrucomicrobia*, unclassified group of *Bacteria* and *Euryarchaeota*, respectively. Moreover, acetic acid had a negative strong association and a high statistical significance to *Synergistetes* (ρ = 0.587, p = 0.006). The *Synergistetes phylum* clearly confirmed a negative association with pro-inflammatory acetic acid, previously seen in healthy subjects and may play an important role in anti-inflammatory process too, however in this case as a consequence of a probiotic administration. *Verrucomicrobia* had also a negative correlation (ρ = 0.486, p = 0.030) to butyric acid, but the later negatively correlated to unclassified *Bacteria* (ρ = 0.498, p = 0.026). SCFAs had a negative correlation to *Synergistetes* and unclassified group of *Bacteria* (ρ = 0.496, p = 0.026 and ρ = 0.517, p = 0.020, respectively). Identified correlations between the mentioned SCFAs and phyla are largely unknown, but they may play a role in the microbiome restoration as a result of probiotic administration.

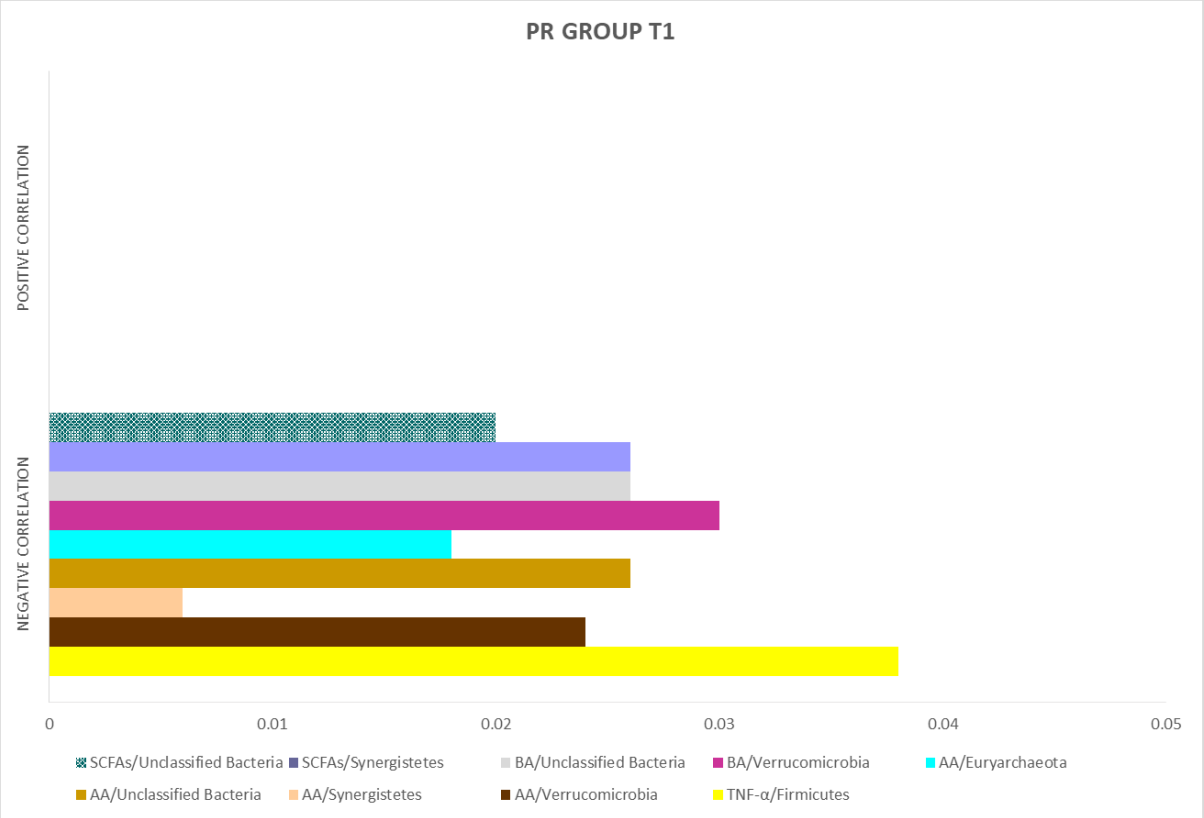

Fig. 3. Statistically significant correlations in the PR group at T1 as a result of probiotic administration. PR: probiotic group; T1: after 3-month treatment; AA: acetic acid; BA: butyric acid; SCFAs: short-chain fatty acids; TNF-α: tumour necrosis factor alpha.

4. Effect of placebo on correlation values in the PL group at T1

When comparing PL group at T0 and T1 (Fig. 4 and Fig. 5, respectively), the phylum *Proteobacteria* and *Verrucomicrobia* confirmed again its important role in CD, already observed in CD group T0. In both PL groups (T0 and T1), *Proteobacteria* positively correlated to acetic, propionic acid and total SCFAs (between $\rho = 0.574$, $p = 0.010$ and $\rho = 0.505$, $p = 0.027$). Furthermore, *Verrucomicrobia* had a strong positive association and a high statistical significance to TNF-α in PL group T0 ($\rho = 0.780$, $p = 0.000135$) and continued to positively correlate to TNF-α in PL group T1 ($\rho = 0.495$, $p = 0.037$). However, *Parcubacteria* again confirmed its important role in CD, while revealing a strong negative association and a high statistical significance ($\rho = 0.590$, $p = 0.010$) to TNF-α in PL group T0. Surprisingly, no statistically significant correlation between the two parameters has been found in PL group T1. Moreover, although not expecting any particular differences between the PL group T0 and the PL group T1, the results in PL group after 3-month placebo treatment revealed some new statistically significant correlations. TNF-α had a negative correlation ($\rho = 0.507$, $p = 0.032$) to *Bacteroidetes* and a positive correlation ($\rho = 0.507$, $p = 0.032$) to *Deinococcus-Thermus*. Furthermore, acetic acid negatively correlated ($\rho = 0.521$, $p = 0.022$) to the group of unclassified *Bacteria*. Propionic acid had a negative ($\rho = 0.471$, $p = 0.042$) correlation to *Synergistetes* and butyric acid had a positive correlation ($\rho = 0.498$, $p = 0.030$) to *Proteobacteria.* Microbiota composition is continuously changing as a result of the complex interplay between environmental factors, such as diet, psychological factors and the host itself. Since there has been a 3-month difference between T0 and T1, microbiota shift was likely to occur even in the PL group.

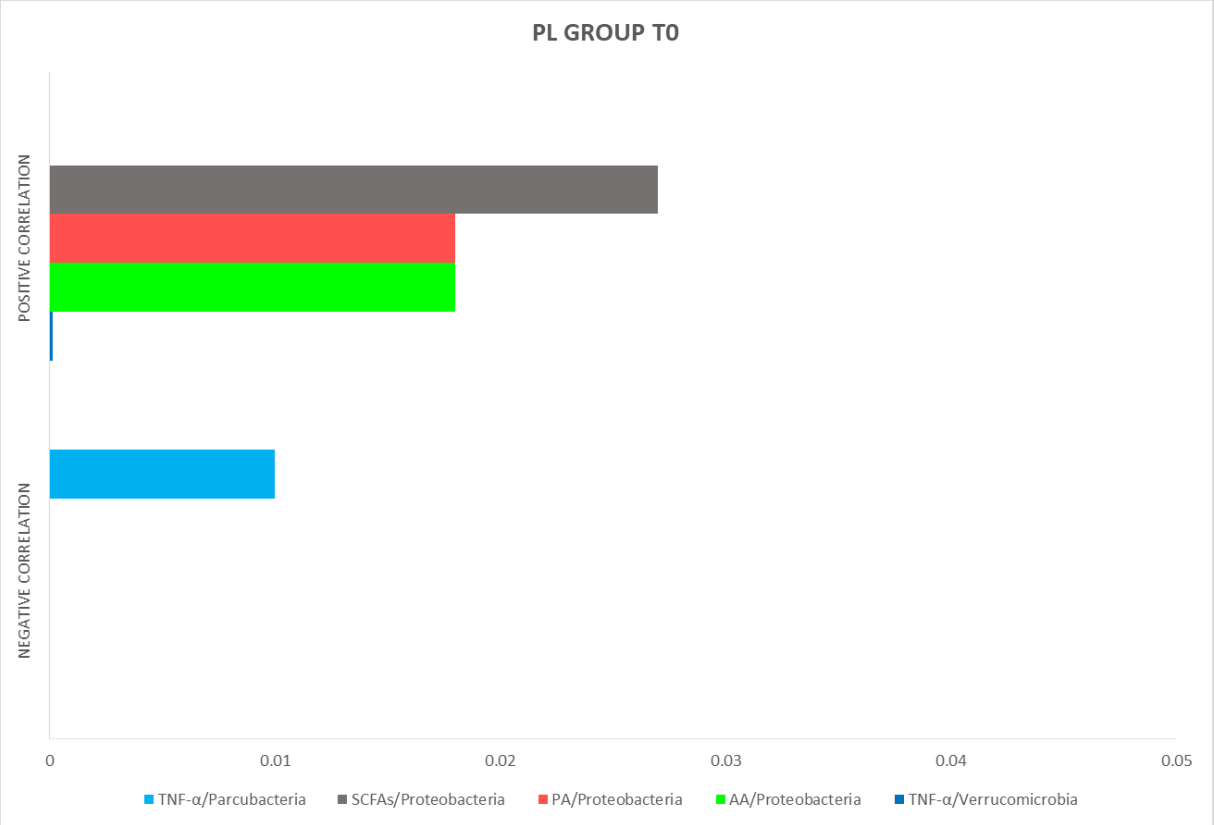

Fig. 4. Statistically significant correlations in PL group at T0. PL: placebo group; T0: enrolment day; AA: acetic acid; PA: propionic acid; SCFAs: short-chain fatty acids; TNF-α: tumour necrosis factor alpha.

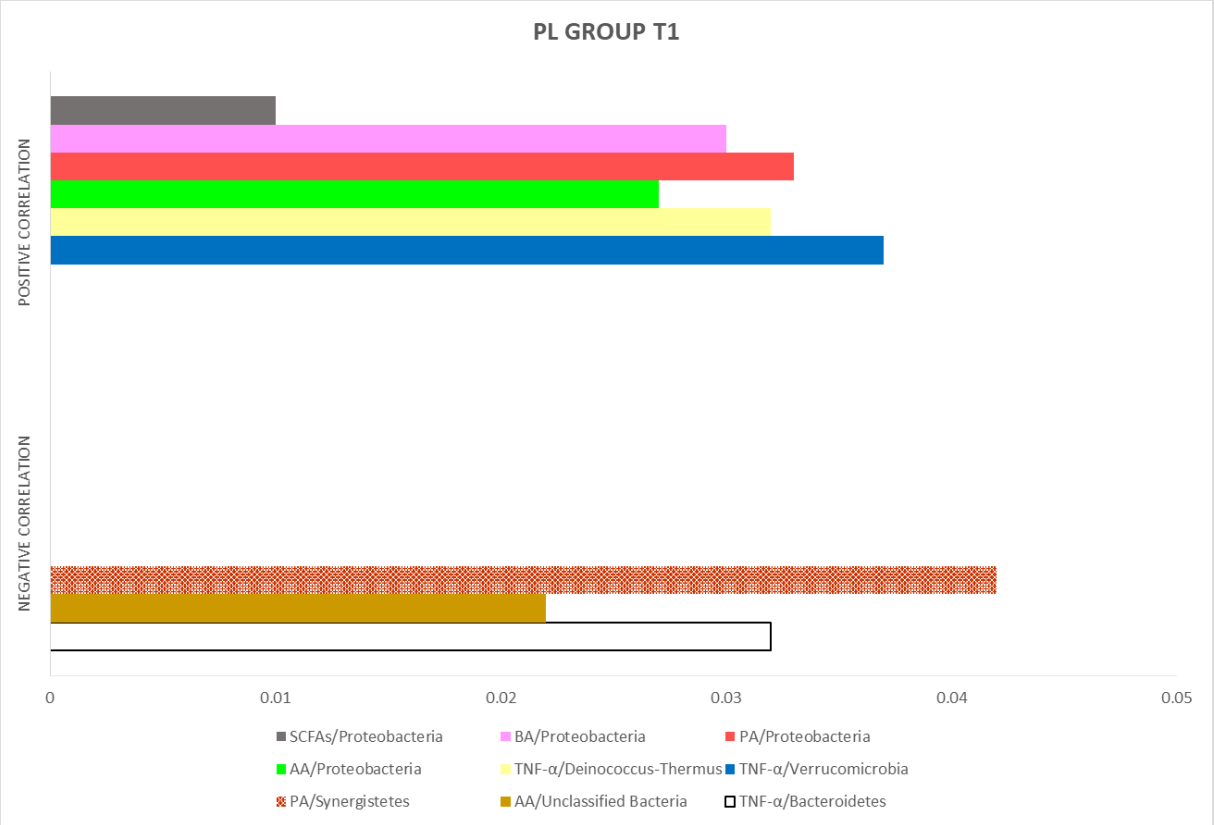

Fig. 5. Statistically significant correlations in PL group at T1 as a result of placebo administration. PL: placebo group; T1: after 3-month treatment; AA: acetic acid; BA: butyric acid; PA: propionic acid; SCFAs: short-chain fatty acids; TNF-α: tumour necrosis factor alpha.

Conclusions

Many physiological changes related to CD have been already described (Fig. 6). However, the complexity of the disease is puzzling with many questions still open. Besides classically documented microbiota changes in the *Firmicutes* phylum, our results have shown that additional phyla such as *Verrucomicrobia*, *Parcubacteria* and some yet unknown phyla belonging to *Bacteria* and *Archaea Kingdom*, may also play an important role in CD-related pathology. Moreover, *Proteobacteria* seems to be responsible for the increase of faecal SCFAs in the disease. In healthy subjects, *Synergistetes* and *Euryarcheota* are present in a minor relative abundance in the human gut system, but they may be additional phyla next to *Firmicutes* contributing to anti-inflammation. Probiotic administration has clearly revealed a negative relationship between *Firmicutes* and pro-inflammatory TNF-α. Moreover, probiotic effect has exposed some new phyla, particularly *Synergistetes*, which negatively correlated to acetic acid and total SCFAs, suggesting a potential role in microbiome restoration. Nevertheless, alterations of microbiota in CD subjects may not be considered exclusively as a consequence of the disease itself, but rather as a part of a complex relationship between many causative factors, including those of diet and psychological nature.

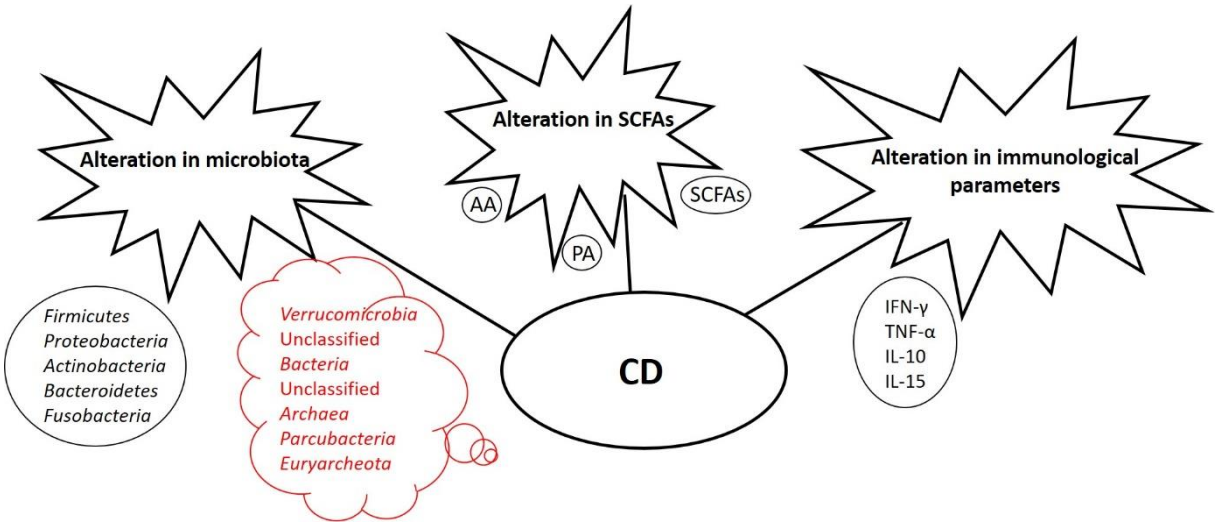

Fig. 6. Schematic representation of the main physiological changes related to CD (shown in black). Potential alterations in microbiota related to CD are shown in red. AA: acetic acid; PA: propionic acid; SCFAs: short-chain fatty acids; IFN-γ: interferon gamma; TNF-α: tumour necrosis factor alpha; IL-10: interleukin 10; IL-15: interleukin 15.

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Supplementary Materials

Supplementary Table 1a. Microbial profile of NGS results in PR group T0.

PHYLA, %	CHILDREN																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Bacteroidetes	31.1	8.3	31.8	42.4	7.4	10.9	39.7	6.3	54.8	57.9	16.1	58.2	60.4	17.7	30.9	47.6	32.7	7.9	16.0	0.7
Firmicutes	49.4	68.9	55.0	24.8	65.1	63.4	40.5	77.1	24.4	29.0	68.2	28.3	21.2	45.7	49.1	36.7	34.0	50.5	58.0	75.5
Actinobacteria	3.0	13.5	0.5	0.4	19.1	2.6	9.8	2.1	2.1	2.8	3.3	0.5	0.1	2.9	13.6	0.7	4.4	2.3	4.3	5.8
Proteobacteria	9.9	1.6	3.6	5.5	1.5	8.3	0.1	4.2	14.7	2.7	6.5	3.1	12.4	19.5	2.9	0.8	7.9	4.2	14.1	1.3
Cyanobacteria/ Chloroplast	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1
Verrucomicrobia	2.8	7.3	0.0	22.7	0.5	11.9	1.6	0.1	2.2	0.5	0.1	1.4	4.1	1.4	0.2	10.7	17.4	31.0	2.3	10.3
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.1	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	2.3	0.2	5.3	2.5	3.8	1.6	4.7	5.9	1.0	4.0	3.4	4.5	1.0	7.5	1.9	1.9	2.1	2.4	3.2	3.8
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.2	0.0	0.1	0.1	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1

Supplementary Table 1b. Microbial profile of NGS results in PR group T1.

PHYLA, %	CHILDREN																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Bacteroidetes	30.9	28.0	25.6	43.9	30.7	30.7	29.3	4.6	53.5	53.6	33.9	26.2	16.5	53.4	32.3	55.6	29.7	19.0	3.7	39.8
Firmicutes	63.1	43.6	66.5	17.6	59.1	55.4	47.4	88.4	34.2	29.6	62.5	52.4	75.6	44.4	45.8	39.4	47.2	77.5	81.2	55.3
Actinobacteria	2.7	14.0	0.4	0.7	4.3	1.3	0.6	4.3	3.3	11.4	2.7	17.5	6.6	0.4	3.2	0.3	7.4	1.6	10.0	0.4
Proteobacteria	1.8	1.7	7.3	7.2	3.6	12.4	21.9	2.2	5.4	3.5	0.4	2.5	0.9	0.9	7.3	2.7	10.3	1.8	4.7	4.2
Cyanobacteria/ Chloroplast	0.3	0.1	0.0	0.1	0.0	0.0	0.2	0.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.4	0.2
Verrucomicrobia	1.0	12.6	0.0	30.3	2.0	0.0	0.4	0.1	3.1	1.8	0.3	1.1	0.0	0.9	10.7	1.7	5.1	0.0	0.0	0.0
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.5	0.1	0.1	0.0	0.0	0.0
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Supplementary Table 1c. Microbial profile of NGS results in PL group T0.

PHYLA, %	CHILDREN																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Bacteroidetes	54.8	2.7	33.7	42.5	55.2	1.9	54.8	23.6	56.6	20.0	49.6	42.2	32.0	1.2	10.7	10.4	33.4	4.7	5.4
Firmicutes	36.0	57.2	45.3	47.9	30.2	64.7	25.6	54.5	40.2	77.1	22.4	49.5	64.0	95.8	22.2	20.0	55.4	13.5	88.9
Actinobacteria	1.6	4.2	19.5	4.6	0.6	2.5	0.4	19.3	0.8	2.0	0.6	1.8	2.2	2.2	0.3	1.8	6.8	5.5	5.3
Proteobacteria	5.4	34.7	1.2	3.6	3.5	30.8	19.0	0.9	2.3	0.6	23.0	6.5	0.9	0.6	1.9	2.8	3.8	23.3	0.3
Cyanobacteria/ Chloroplast	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.1	0.0	0.2	0.0
Verrucomicrobia	2.1	0.1	0.0	0.3	10.3	0.0	0.0	1.4	0.0	0.0	4.3	0.0	0.8	0.0	64.0	64.4	0.4	52.7	0.0
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	0.0	0.1	0.1	0.0	0.2	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Supplementary Table 1d. Microbial profile of NGS results in PL group T1.

PHYLA, %	CHILDREN																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Bacteroidetes	25.7	1.1	14.7	37.4	22.6	58.9	59.5	35.6	71.5	21.8	57.2	51.7	1.8	40.9	1.6	1.0	72.4	12.9	4.4
Firmicutes	60.8	49.9	28.7	45.9	55.2	36.3	32.4	52.4	26.0	24.6	35.0	42.7	85.0	54.3	28.8	51.9	26.1	9.1	73.2
Actinobacteria	7.4	1.7	5.1	7.0	2.1	1.7	0.9	8.3	0.3	1.0	0.2	0.8	1.8	0.7	0.3	0.7	0.6	0.2	21.3
Proteobacteria	1.3	47.2	51.4	8.1	2.5	3.1	5.3	3.1	1.8	16.0	7.1	4.5	8.5	0.8	0.3	45.3	0.7	0.3	0.6
Cyanobacteria/ Chloroplast	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.7	0.0	0.0	0.0	0.0	0.0	0.0
Verrucomicrobia	4.9	0.0	0.0	0.4	16.6	0.0	1.7	0.2	0.1	36.3	0.3	0.0	0.0	3.2	68.9	1.0	0.0	77.4	0.1
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	0.0	0.0	0.0	0.0	0.6	0.1	0.1	0.2	0.1	0.1	0.2	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.2
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Supplementary Table 1e. Microbial profile of NGS results in HC group T0.

PHYLA, %	CHILDREN														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Bacteroidetes	55.9	21.5	15.6	0.6	5.7	18.3	53.9	17.5	30.7	6.4	18.5	8.2	28.7	5.0	10.3
Firmicutes	37.6	42.3	73.3	9.4	71.2	58.3	31.7	73.9	59.4	71.4	57.5	77.4	18.6	67.0	80.6
Actinobacteria	2.3	2.1	5.9	0.2	20.3	9.2	1.9	2.0	4.0	9.4	18.7	12.5	3.0	11.4	7.6
Proteobacteria	3.2	32.8	1.6	89.7	2.5	12.1	10.8	2.5	4.9	7.9	1.9	1.3	2.1	1.2	1.2
Cyanobacteria/ Chloroplast	0.0	1.1	0.5	0.0	0.2	1.5	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0
Verrucomicrobia	0.9	0.1	3.0	0.0	0.0	0.0	1.0	3.8	0.9	0.8	2.3	0.2	47.5	15.0	0.2
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.1	0.1
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	0.0	0.0	0.0	0.0	0.0	0.1	0.3	0.1	0.0	0.1	0.1	0.0	0.1	0.2	0.0
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.4	0.1	0.0	0.1	3.9	0.9	0.3	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Supplementary Table 1f. Microbial profile of NGS results in CD group T0.

PHYLA, %	CHILDREN																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Bacteroidetes	31.1	8.3	31.8	42.4	7.4	10.9	39.7	6.3	54.8	57.9	16.1	58.2	60.4	17.7	30.9	47.6	32.7	7.9	16.0	0.7
Firmicutes	49.4	68.9	55.0	24.8	65.1	63.4	40.5	77.1	24.4	29.0	68.2	28.3	21.2	45.7	49.1	36.7	34.0	50.5	58.0	75.5
Actinobacteria	3.0	13.5	0.5	0.4	19.1	2.6	9.8	2.1	2.1	2.8	3.3	0.5	0.1	2.9	13.6	0.7	4.4	2.3	4.3	5.8
Proteobacteria	9.9	1.6	3.6	5.5	1.5	8.3	0.1	4.2	14.7	2.7	6.5	3.1	12.4	19.5	2.9	0.8	7.9	4.2	14.1	1.3
Cyanobacteria/ Chloroplast	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1
Verrucomicrobia	2.8	7.3	0.0	22.7	0.5	11.9	1.6	0.1	2.2	0.5	0.1	1.4	4.1	1.4	0.2	10.7	17.4	31.0	2.3	10.3
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.1	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	2.3	0.2	5.3	2.5	3.8	1.6	4.7	5.9	1.0	4.0	3.4	4.5	1.0	7.5	1.9	1.9	2.1	2.4	3.2	3.8
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.2	0.0	0.1	0.1	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1

Supplementary Table 1f. Microbial profile of NGS results in CD group T0 (continued).

PHYLA, %	CHILDREN																		
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Bacteroidetes	54.8	2.7	33.7	42.5	55.2	1.9	54.8	23.6	56.6	20.0	49.6	42.2	32.0	1.2	10.7	10.4	33.4	4.7	5.4
Firmicutes	36.0	57.2	45.3	47.9	30.2	64.7	25.6	54.5	40.2	77.1	22.4	49.5	64.0	95.8	22.2	20.0	55.4	13.5	88.9
Actinobacteria	1.6	4.2	19.5	4.6	0.6	2.5	0.4	19.3	0.8	2.0	0.6	1.8	2.2	2.2	0.3	1.8	6.8	5.5	5.3
Proteobacteria	5.4	34.7	1.2	3.6	3.5	30.8	19.0	0.9	2.3	0.6	23.0	6.5	0.9	0.6	1.9	2.8	3.8	23.3	0.3
Cyanobacteria/ Chloroplast	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.1	0.0	0.2	0.0
Verrucomicrobia	2.1	0.1	0.0	0.3	10.3	0.0	0.0	1.4	0.0	0.0	4.3	0.0	0.8	0.0	64.0	64.4	0.4	52.7	0.0
Candidatus Saccharibacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Planctomycetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deinococcus-Thermus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acidobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
Parcubacteria	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synergistetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chlamydiae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Armatimonadetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microgenomates	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tenericutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spirochaetes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elusimicrobia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Bacteria	0.0	0.1	0.1	0.0	0.2	0.0	0.0	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0
Euryarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Crenarchaeota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
unclass_Archaea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Paper 5

Efficacy of the treatment with *Bifidobacterium breve* B632 and BR03 in paediatric obesity: a cross-over double blind randomized controlled trial.

This work has been conducted in collaboration with the Department of Health Sciences (University of Piemonte Orientale, Novara), Department of Translational Medicine (University of Piemonte Orientale, Novara), Interdisciplinary Research Centre of Autoimmune Diseases (University of Piemonte Orientale, Novara), R&D Biolab (Novara), CPO Piemonte.

This paper has not been completed yet and it needs finalization prior to publication

Abstract

Studies show that lean and overweight individuals have a different gut microbiota composition that can be involved in pathogenic mechanisms linked to obesity. Few studies have been done in humans, in particular in paediatrics, to evaluate how the manipulation of the gut microbiota through probiotic administration could impact on obesity and its associated comorbidities. The objective of this study is to clarify if *Bifidobacterium breve* B632 and *Bifidobacterium breve* B03 are able to rescue the metabolic homeostasis in obese children. This is a cross-over double blind randomized clinical trial concluded on 100 obese children and adolescents (6-18 years). The subjects, to whom an isocaloric diet in the Mediterranean style and physical activity were applied, were randomly treated with a probiotic formulation consisting of $3x10^{\wedge 8}$ CFU of *B. breve* B632 and *B. breve* B03, or placebo once daily for 8 weeks. After a 4-week wash-out period, individuals were subjected to cross-over; those who has previously taken the probiotic were given the placebo and vice versa for 8 weeks. Clinical, biochemical and faecal samples were then analysed.

After the first 8 weeks of treatment, subjects receiving the probiotic supplement showed significantly lower values of basal insulin, HOMA-IR and acetic acid and significantly higher values of ISI compared to the placebo one. In addition, the probiotic group showed a more marked reduction of glycaemia at 0 and 120 min after OGTT, BMI, BMI Z-score and waist circumference and an increase in *Bifidobacterium* spp. and *B. breve* counts with respect to the placebo one. However, the positive action of the probiotic intervention on body composition, glyco-insulinemic metabolism and gut microbial profile documented after the first 8 weeks of treatment, was not observed in the second phase of the study.

The treatment with *B. breve* B632 and *B. breve* B03 can significantly ameliorate glucose metabolism, influencing the body composition and the gut microbiota of obese children and adolescents following a diet therapy. The confirmation of these data with further analyses, could have a crucial impact on the care strategies for paediatric obesity.

Keywords: paediatric obesity, gut microbiota, probiotics, *Bifidobacterium breve*, BMI, glycaemia, insulin

Introduction

Paediatric obesity represents one of the greatest public health challenges of the 21st century. In 2016, according to the World Heart Organization (WHO), 124 million children and adolescents aged 5-19 and 671 million adults were estimated obese. Moreover, data from 2018 collected by the WHO showed that 213 million children and 1.9 billion adults are in an overweight condition.

Childhood obesity tends to persist even in adulthood, associated with a high risk of developing chronic comorbidities, such as diabetes mellitus, cardiovascular, respiratory, and osteoarticular pathologies, psychosocial and cancer disorders including those of the colon, breast and endometrium (Reilly and Kelly, 2011). In addition, the socio-economic impact deriving from obesity has an important relevance. The burden of disease attributable to excess weight is responsible for about 4 million deaths worldwide (GBD 2015 Obesity Collaborators, 2017). Moreover, healthcare costs aimed at treating physical and psychosocial complications and indirect costs, deriving from reduced productivity and absence from work, resulted increased (Dee et al., 2014). The WHO Member States with the Action Plan on Childhood Obesity 2014-2020 (February 2014) have adopted the common goal of developing information, social, environmental and political interventions of health and physical activity education for obesity prevention.

Obesity is a chronic and multifactorial condition characterized by an excessive development of adipose tissue, attributable, particularly in childhood, to tissue hyperplasia with an increase of adipocytes (Bray, 2004). Several complications are attributable to paediatric obesity: hypertension (Genovesi et al., 2010; Lo et al., 2014), dyslipidemia (Patricia et al., 2014), alterations of the glucan metabolism (Haemer et al., 2014), endocrine disorders (Li et al., 2017; Anderson et al., 2014; Rojas et al., 2014), altered respiratory physiology (Valerio et al., 2018; Fiorino and Lee, 2009; Santamaria et al., 2012), gastroenteric disorders (Hardy et al., 2016; Davies et al., 2012; Koebnick et al., 2012), orthopedic disorders (Aversano et al., 2016; Bout-Tabaku et al., 2015; Stolzman et al., 2015), psychosocial disorders (Sagar and Gupta, 2018; Fox et al., 2016). The imbalance between caloric intake and energy expenditure is characterized by a complex relationship between multiple genetic, environmental and behavioural factors (Ang et al., 2013).

Some studies conducted on murine models highlighted the connection between the excess of adiposity and the gut microbiota. Bäckhed et al. (2004) showed that the total body fat of germ-free (GF) mice was 40% lower than conventional mice, although their food consumption was higher. Moreover, they documented that the transplantation of the intestinal microbiota in GF mice induced an increase in adiposity after 2 weeks 60%. In other experiments, the colonization of gnotobiotic lean mice with gut microorganisms from genetically obese mice (ob / ob), compared to those from lean mice, resulted in a higher ability to extract calories from food and a significantly higher increase in body fat (Davis, 2016).

Also human studies have shown this interaction, evidencing alterations in microbial gut ecology. Ignacio et al. (2016) from the analysis of 84 faecal samples of overweight, obese and lean children showed that the body mass index (BMI) was positively correlated with *Bacteroides fragilis* and *Lactobacillus* levels and negatively correlated with *Bifidobacterium* levels. Nadal et al. (2009) described a significant reduction of *Clostridium histoliticum*, *Clostridium coccoides* and *Eubacterium rectal* and in an increase of *Bacteroides* and *Prevotella* in overweight and obese adolescents following a calorie-restricted diet in association with physical activity. Santacruz et al. (2009) observed an increase in *B. fragilis* and *Lactobacillus* levels and a decrease of *Bifidobacterium longum* and *C. coccoides* with nutritional and physical interventions. In addition, a

prospective follow-up study by Kalliomaki et al. (2008) highlighted that alterations of the intestinal microbiota in the early stage of life were also associated to a higher risk to develop overweight or obesity during life. In particular, the number of bifidobacteria in faecal samples during infancy was higher in children remaining normal weight than in children becoming overweight, whereas *Staphylococcus aureus* recovery was higher in children becoming overweight than in children remaining normal weight.

The gut microbiota contributes to the extraction of energy from foods through the production of short chain fatty acids (SCFAs). SCFAs are able to stimulate the hepatic lipogenesis by promoting the expression of ChREBP and SREBP-1, proteins that control the activity of enzymes acetyl-CoA carboxylase and the fatty acid synthase (Bäckhed et al., 2004).

A dysbiotic gut microbiota, associated to a hyperlipidic diet, can promote a chronic inflammatory response that may contribute to obesity pathogenesis (Sun et al., 2018). One of the mechanisms involved is the modulation of the lipoprotein lipase (LPL) activity, via the suppression of its antagonist enzyme, the Fasting-Induced Adipose Factor (FIAF). This mechanism potentially stimulates host weight gain by impairing triglyceride metabolism and promoting fat storage (Sun et al., 2018, Parekh et al., 2014). A dysbiotic microbiota also contributes to the accumulation of body fat by inhibiting the AMPK (Adenosine Monophosphate-Activated Protein Kinase), an enzyme involved in the oxidation of fatty acids in the liver and in the skeletal muscle (Parekh et al., 2014). An interesting study conducted by Vrieze et al. (2012) demonstrated that after the infusion of microbiota from lean donors, insulin sensitivity of obese recipients increased together with levels of butyrate-producing intestinal microbiota.

Several studies on animal and human models highlighted the positive effects on the weight status and metabolic disorders associated to probiotic treatments. However, it must be emphasized that the randomized controlled trials that have been conducted on obese adult subjects are few, and even less in paediatric patients, and short-term.

In a multicentre, randomized, placebo-controlled study the administration of *Lactobacillus gasseri* for 12 weeks led to a reduction of visceral and subcutaneous fat by 4.6% and 3.3%, respectively, 1.4% decrease in body weight and 1.5% in BMI (Kadooka et al., 2010). Beneficial effects of lactobacilli on obesity-related metabolic disorders are suggested by the ability to reduce fasting blood glucose, HbA1c levels and insulin resistance in patients with type 2 diabetes mellitus (Razmpoosh et al., 2016; Mazloom et al., 2013). The commercial probiotic formulation VSL#3 was shown to be effective in improving lipid profile, insulin sensitivity and inflammatory parameters (Rajkumar et al., 2014). Moreover, some clinical trials revealed promising effects of probiotics in improving liver function, fat metabolism and insulin resistance in patients with obesity related non-alcoholic fatty liver disease (Vajro et al., 2011; Mykhalchyshyn et al., 2013).

Some works evidenced a potential anti-obesity property associated to *B. breve* strains. The treatment with *B. breve* B-3 revealed significantly lowered fat mass and improved blood parameters related to liver functions and inflammation in adults with a tendency for obesity treated (Minami et al., 2015) . Aloisio et al. (2018) showed that the administration of *B. breve* B632 and Br03 in children born by caesarean section, at high risk to develop obesity later in life (Magne et al., 2017; Kuhle et al., 2015), led to a lower catch-up growth in weight, reducing the risk of metabolic disturbances later in life.

The manipulation of the microbiota by probiotic supplementation is a possible, safe and well tolerated approach to obesity, but it is necessary to conduct further studies about targeted probiotics,

treatment duration and size of study population in paediatric subjects to better understand the effects deriving from a probiotic intervention.
This study aims to assess the impact of dietary supplementation of two *B. breve* strains, B632 and BR03 in obese children and adolescents on a dietetic therapy, on the clinical and metabolic profile and on gut microbiota composition.

Materials and methods

Study population

One hundred obese children and adolescents were recruited at the Auxology and Endocrinology Section of the Paediatric Clinic of the Charity Major Hospital of Novara, from November 2013 to October 2017. The study was approved by the Inter-company Ethics Committee (CE165/13) and registered on Clinical Trial (NCT03261466).
The inclusion criteria were:

- Age between 6 and 18 years
- BMI compatible with obesity according to IOTF (International Obesity Task Force) criteria (Cole and Lobstein, 2012)
- HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) > 2.5 or insulin > 15µU/ml
- Puberty stage ≥2 according to Tanner staging (Tanner, 1962)

The exclusion criteria were:

- Contraindications such as allergy to the components of the drug
- Obesity derived from genetic (Prader Willi syndrome, Down syndrome), metabolic (Laurence-Biedl syndrome) and endocrine (Cushing's syndrome, hypothyroidism) diseases
- Hepatic-gastrointestinal chronic disorders
- Ongoing therapy for chronic systemic diseases
- Previous administration (3 months earlier) with antibiotics, probiotics or prebiotics

Phase 1
Subjects were randomized into 2 homogeneous groups by number and sex with a 1:1 ratio to probiotic or placebo treatment for 2 weeks. At the time of recruitment, an isocaloric diet in the Mediterranean style was recommended with 55-60% of carbohydrates, 25-30% of fats and 15% of proteins as recommended by the Endocrine Society Guidelines (Holick et al., 2011) and by the Italian Standards for Care Obesity of the Italian Obesity Society. Furthermore, all patients were given general indications on physical exercise, i.e. 30-60 minutes of daily aerobic activity and a sport practice at least 1-2 times a week.

Phase 2
After a 4-week wash-out period the individuals were subjected to a cross-over; those who has previously taken the probiotic were given the placebo and vice versa for 8 weeks (**Fig. 1**). The general recommendations on diet and physical activity were not modified.

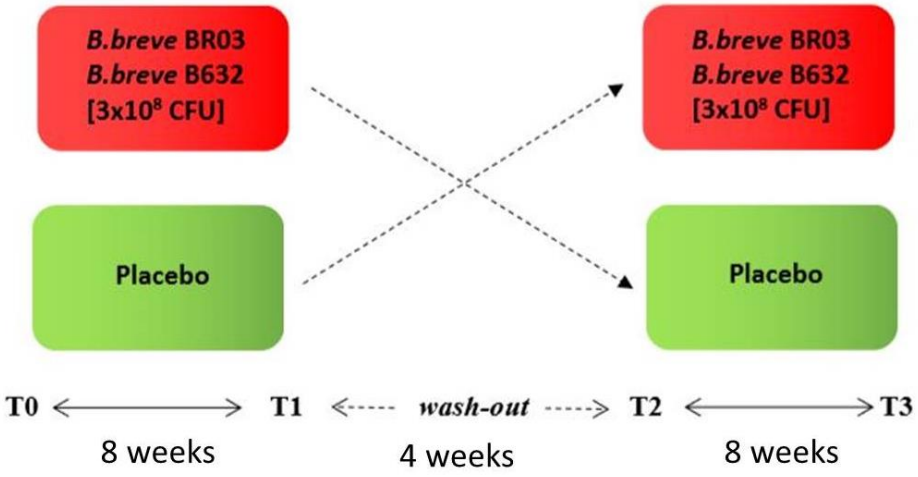

Fig. 1. Study design of the placebo-controlled crossover clinical trial

Probiotic treatment

Bifibaby ® (Probiotical S.p.A., Novara, Italy), containing the two bacterial strains *B. breve* B632 (DSM 24706) and *B. breve* BR03 (DSM 16604) prepared in an oily suspension, was administered at a daily dosage of 15 drops containing 3 x 10^8 CFU. The placebo was composed of the same amount in weight of malt dextrin.

Patients underwent anamnestic evaluation, clinical-auxological examination and blood tests at the time of recruitment (T0), at the end of the first 8 weeks of treatment (T1), after 4 weeks of wash-out (T2) and after the second 8-week treatment (T3). A random number generator (Lehmer generator) was used to randomize patients to probiotic treatment or placebo.

Clinical monitoring

Auxological evaluation (T0, T1, T2, T3)

At each visit, the patients were subjected to the following assessments:

- Height, measured with the Harpenden statimeter with approximation to the nearest 0.5 cm and classification according to the Italian growth curves (Cacciari et al., 2006).
- Weight, measured with manual scale with approximation to the nearest 0.1kg and classification according to Italian growth curves (Cacciari et al., 2006).
- BMI (kg / m2)
- Calculation of the BMI percentile by sex and age and classification according to the IOTF curves (Cole and Lobstein, 2012) and according to the Italian growth curves (Cacciari et al., 2006).
- Calculation of the BMI Z-score with the L, M, S method according to the model proposed by Cole and Lobstein (2012) and using the Italian growth curves (Cacciari et al., 2006).
- Waist circumference, measured with a flexible meter applied in the area between the ribs and the iliac crest, in an upright position, at the end of a normal expiration, with registration at 0.1cm.

Biochemical Evaluation (T0, T1, T2, T3)

After at least 12 hours of night fasting the subjects were subjected to the evaluation of:

- Basal glycaemia (glycaemia 0') and glycaemia measured after 120 min (glycaemia 120') from the Oral Glucose Tolerance Test (OGTT) (1.75 g / kg of glucose per os at time 0 ', maximum dose 75 g)
- Basal insulin levels (insulin 0') and insulin levels measured after 30 min (insulin 30') from OGTT
- HOMA-IR, calculated from calculated from the parameters obtained by the OGTT
- The insulin sensitivity (ISI) defined by the Matsuda Index, calculated from the parameters obtained by the OGTT
- Triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol
- IL-6 (pg / mL), IL-10 (pg / mL), TNF-α (pg / mL)
- LPS (ng / mL)

Glycaemia, insulin levels, HDL and LDL cholesterol and triglycerides were measured with Siemens Advia 2400® (Healthcare Diagnostics, Deerfield, USA).

The plasma levels of IL-6 and IL-10 were measured using Human Interleukin ELISA kit (Thermo Fisher Scientific, Waltham, USA); TNF-α were was detected with Human TNF-α kit (ELISA Thermo Fisher Scientific, Waltham, USA). Plasma LPS levels were measured using the Enzyme-linked Immunosorbent Assay Kit for Lipopolysaccharide (Thermo Fisher Scientific, Waltham, USA). The blood samples were collected from fasting subjects, centrifuged at 3000 rpm for 15 minutes in refrigerated conditions (+ 4 ° C) and stored at -20 ° C until the time of analysis and processed according to standard procedure.

Gut microbiota analysis

DNA extraction from faecal samples

DNA was extracted from 200 mg of faeces (preserved at -80 °C after collection) using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) with a supplementary incubation at 95 °C for 10 min of the stool sample with the lysis buffer, to enhance the bacterial cell rupture (Aloisio et al. 2014). Extracted DNA was stored at -20 °C. The purity of DNA was determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite®200 PRO NanoQuant, Tecan, Mannedorf, Switzerland) and the concentration was evaluated by Qubit® 3.0 Fluorometer (Invitrogen, Life Technologies, CA, USA).

Absolute quantification of selected microbial groups using quantitative PCR (qPCR)

Quantification of selected microbial groups, i.e. *Bidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis* group (comprising the most abundant species in human *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. vulgatus*), *B. breve* and *Escherichia coli* was performed with qPCR on DNA extracted from stool samples. The assays were carried out with a 20 µL PCR amplification mixture containing 10 µL of Fast SYBR® Green Master Mix (Applied Biosystems, Foster city, CA, USA) optimized concentrations of primers, molecular grade H_2O and 2 µL DNA obtained from faecal samples at a concentration of 2.5 ng/µL. *B. breve* analysis was performed using a TaqMan assay containing 12.5 µL of Universal TaqMan master mix (Applied Biosystems, Foster city, CA, USA), 300nM of each primers and 100 nM of probe labeled with the

5’ reporter dye 6-carboxyfluorescein and the 3’quencher NFQ-MGB (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). 40 PCR cycles were performed. Primers and PCR conditions are reported in **Table 1** and **2**.

Table 1. Primer sequences and qPCR conditions used in the different assays.

Microorganism target	Primer	Sequence (5’-3’)	Amplicon length(bp)	References
E. coli	Eco-F Eco-R	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	340	Malinen et al, 2003
Bifidobacterium spp.	Bif-F Bif-R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	243	Penders et al., 2005
Lactobacillus spp.	Lac-F Lac-R	GCAGCAGTAGGGAATCTTCCA GCATTYCACCGCTACACATG	349	Rinttila et al., 2003
B. fragilis group	Bfra-F Bfra-R	CGGAGGATCCGAGCGTTA CCGCAAACTTTCACAACTGACTTA	92	Penders et al., 2006
B. breve	F _IS R_IS P_IS	GTGGTGGCTTGAGAACTGGAT AG CAAAACGATCGAAACAAACACTAAA TGATTCCTCGTTCTTGCTGT	118	Haarman et al., 2005

Table 2. qPCR amplification protocols and primer concentrations

Taget Bacteria	Initial denaturation	Denaturation	Annealing t (°C)	N. cycles	Fw	Rev
E.coli	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	400 nM	400 nM
Bifidobacterium spp.	95°C – 20sec	95°C - 3 sec	60°C - 35sec	40	200 nM	300 nM
Lactobacillus spp.	95°C – 20sec	95°C - 3 sec	63.5°C - 30 sec	40	200 nM	200 nM
B. fragilis group	95 °C – 20 sec	95 °C – 3 sec	60 °C – 30 sec	40	300 nM	300 nM
B. breve	95 °C – 20 sec	95 °C – 3 sec	60 °C – 30 sec	40	300 nM	300 nM

The primer concentrations were optimized through primer optimization matrices in a 48-well plate and estimating the best Ct/Rn ratio. The different primers were also checked for their specificity using the database similarity search program nucleotide-nucleotide BLAST (Altschul e al., 1990). Moreover, to evaluate the specificity of amplification, melting curve analysis was performed after the last cycle of each amplification. Collected data were then converted to obtain the number of bacterial (Log CFU/g faeces) in accordance with the rRNA copy number available at the rRNA copy number database (Lee et al., 2009). Standard curves were constructed using 16S rRNA PCR products of type strains of each target microorganism; the standard microorganisms used were *B. breve* ReO2, *L. plantarum* ATCC 14917, *B. fragilis* DSM 2151, *B. breve* B632 DSM 20213, *C. sporogenes* ATCC 319, *E. coli* ATCC 8739. PCR products were purified with a commercial DNA purification system (NucleoSpin® Extract II kit, MACHEREY-NAGEL GmbH & Co. KG, Germany) and the concentration measured spectrophotometrically at 260 nm. Serial dilutions were performed and 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 copies of the gene per reaction were used for calibration. Sample reactions were conducted in triplicate, with a negative control per each reaction.

Short Chain Fatty Acids (SCFA) analysis

The faecal samples analysed belonged to the phase 1 of the study (T0-T1). A Gas Chromatographic analysis with Flame Ionization Detector (FID) was performed. Stool samples were weighed (1 g), suspended in 5 ml of distilled water and homogenized with a horizontal stirrer for 5 minutes. The samples were acidified with HCl (5 M) to a pH ~ 2 and subsequently centrifuged (4000 rpm x 20 min). 980 µL of supernatant was added to 20 µL of methacrylic acid (2.5 mMol / ml). SCFAs content (acetic acid, propionic acid and butyric acid) was determined using the Hewlett Packard 5890 Series II gas chromatograph equipped with FID by injecting 1µL of each sample in a Supelco SPBTM 30m x 0.25mm x 0.25 mm capillary column. The results were processed using a Hewlett Packard 3396 Series II integrator (Tangerman and Nagengast, 1996).

Statistical analysis

Tests for normality and equality of variance (Shapiro and Levene tests) were performed. Statistical significance was evaluated with one-way ANOVA; two-way repeated measure ANOVA comparing different variance-covariance models was used to evaluate time-treatment interactions. Non-normal and non-homoscedastic data were analysed by non-parametric Kruskall-Wallis test.

Results

Among the 100 subjects recruited, 10 (7 probiotic and 3 placebo) did not adhere to phase 2 of the study and 9 (5 probiotic and 4 placebo) did not complete the phase 1. No adverse events occurred during the study and the compliance with the treatment was adequate.

Clinical, metabolic and microbial characteristics at the time of recruitment (T0)

There were no significant differences between the probiotic and the placebo group at the time of recruitment except for *E. coli* count, which was higher in the placebo group. The clinical and metabolic characteristics of the population, divided into the probiotic group the placebo group at T0, are shown in **Table 3**.

Table 3. Clinical and metabolic characteristics of the probiotic and the placebo group at the time of recruitment (T0). BMI: body mass index; WC: waist circumference; HOMA-IR: homeostatic model assessment for insulin resistance; ISI: insulin sensitivity index; HDL: high density lipoprotein; LDL: low density lipoprotein; IL-6: interleukin 6; IL-10: interleukin 10; TNF-α: tumour necrosis factor alpha; LPS: lipopolysaccharide. All data are expressed as mean ± standard deviation.

Variable	Probiotic group (T0)	Placebo group (T0)
Age (years)	12.07 ± 2.57	11.74 ± 2.84
BMI (kg/m2)	30.55 ± 4.66	30.86 ± 5.40
BMI z-score (kg/m2)	2.32 ± 0.48	2.43 ± 0.54
WC (cm)	95.52 ± 15.95	94.63 ± 10.83
Glycaemia 0' (mg/dl)	87.9 ± 7.07	88.82 ± 6.51
Glycaemia 120' (mg/dl)	112.37 ± 20.38	109.33 ± 20.35
Insulin 0' (mIU/l)	23.72 ± 11.01	25.20 ± 13.18
Insulin 30' (mIU/l)	123.93 ± 79.86	126.39 ± 95.88
HOMA-IR	5.18 ± 2.51	5.56 ± 3.06
ISI	2.81 ± 1.59	2.71 ± 1.35
Total cholesterol (mg/dl)	142.04 ± 29.46	145.25 ± 26.82

HDL cholesterol (mg/dl)	40.09 ± 9.04	41.75 ± 8.13
LDL cholesterol (mg/dl)	84.27 ± 24.56	86.92 ± 22.84
Triglycerides (mg/dl)	88.37 ± 50.12	82.90 ± 36.03
IL-6 (pg/dl)	2.32 ± 1.50	2 ± 0.9
IL-10 (pg/dl)	4.27 ± 4.63	4.51 ± 4.73
TNF-α (pg/dl)	10.68 ± 4.64	11.03 ± 4.54
LPS (pg/dl)	1.94 ± 0.67	1.96 ± 0.69
***E. coli* (Log CFU/g)**	6.09 ± 1.06	6.62 ± 0.84
***B. fragilis* (Log CFU/g)**	8.89 ± 0.84	9.07 ± 0.96
***B. breve* (Log CFU/g)**	4.09 ± 1.06	4.37 ± 0.97
***Bifidobacterium* spp. (Log CFU/g)**	8.17 ± 1.17	8.18 ± 0.98
***Lactobacillus* spp. (Log CFU/g)**	5.91 ± 0.87	5.72 ± 1.17

Clinical, metabolic and microbial characteristics in the phase 1 (T0-T1)

For all the subjects, BMI (28.84 ± 4.77 vs 29.54 ± 5.29 kg/m2), BMI Z-score (2.05 ± 0.58 vs 2.22 ± 0.61 kg /m2), waist circumference (91.7 ± 12.01 vs 94.42 ± 15.68 cm), insulin 0' (18.65 ± 10.26 vs 25.77 ± 24.13 mUI /l), HOMA-IR (4.03 ± 2.5 vs 5.73 ± 5.6), IL-10 (3.78 ± 3.46 vs 4.39 ± 5.15 pg / mL) and the faecal concentration of *Lactobacillus* spp. (5.82 ± 1,04 vs 5.72 ± 1.22 Log CFU/g) (**Table 4**) were reduced at T1. At T1 the probiotic group showed significantly lower values of insulin 0' (18.65 ± 10.26 vs 25.77 ± 24.13 mIU /l, p <0.03) (**Fig. 2A**) and HOMA-IR (4.03 ± 2.50 vs 5.73 ± 5.60, p <0,03) (**Fig. 2B**) and significantly higher values of ISI (3.64 ± 1.98 vs 2.86 ± 1.60, p <0.04) (**Fig. 2C**) compared to the placebo one. In addition, the probiotic group showed a more marked reduction glycaemia 0' (**Fig. 2D**) 120' (**Fig. 2E**), in BMI (**Fig. 3A**), BMI Z-score (**Fig. 3B**), waist circumference (**Fig. 3C**), although not statistically relevant, with respect to the placebo one. No differences were detected in the insulin 120' (data not shown). Regarding the bacterial data, *Bifidobacterium* spp. and *B. breve* counts increased more, although not with a statistical significance, in the probiotic group with respect to the placebo one (**Fig. 3A** and **3B, Table 5**).
No significant differences were observed in inflammatory chemokines, lipidic profile and in the other bacterial groups (data not shown).
At T1 the analysis of SCFA showed a significant reduction in acetic acid (0.25 ± 0.48 vs 0.24 ± 0.48 ppm, p <0.01) (**Fig. 5**), 2-methylbutanoatodimethyl (0.14 ± 0.16 vs 0.08 ± 0.16 ppm, p <0.01), and 3-methylbutanoatodimethyl (0.2 ± 0.23 vs 0.09 ± 0.17, p <0.01) (data not shown) in the probiotic group compared to the placebo one.

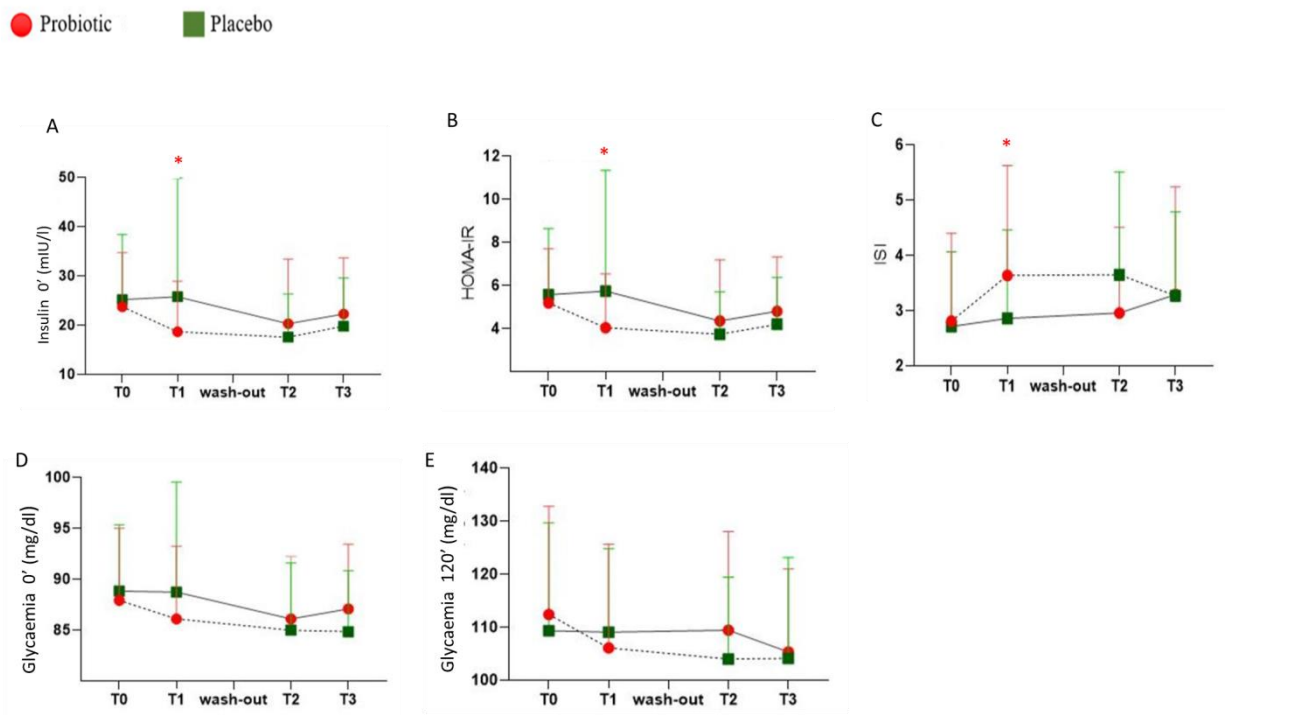

Fig. 2. Variations of glyco-insulinemic parameters in the probiotic and the placebo group during the study: insulin levels at 0' of OGTT (**A**), HOMA-IR (**B**), insulin sensitivity index (ISI) (**C**), glycaemia at 0' (**D**) and 120' from OGTT (**E**). "*" indicates $p<0.05$

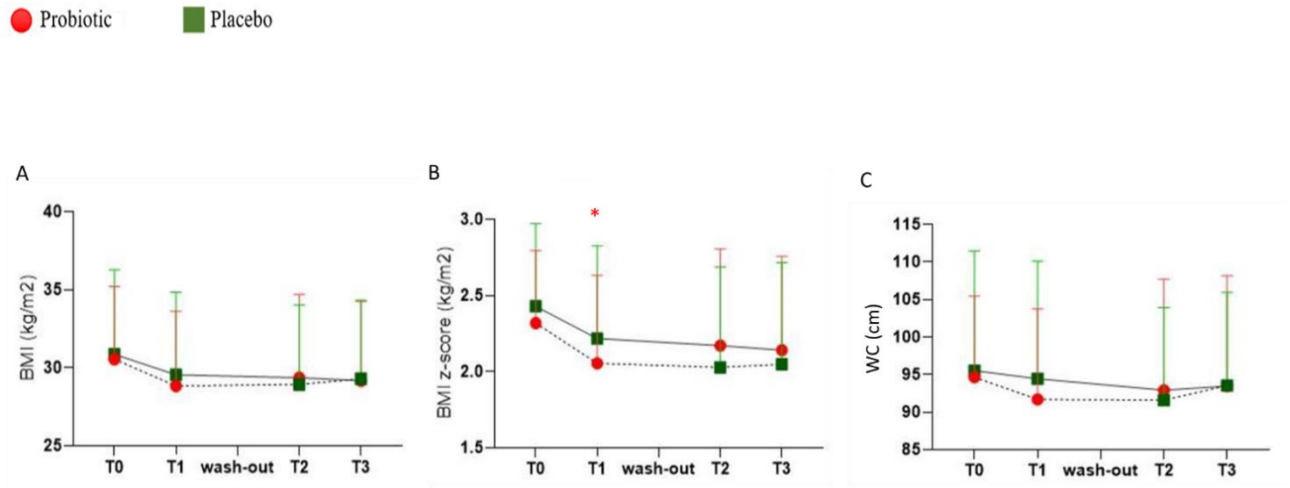

Fig. 3. Variations of auxological parameters in the probiotic and the placebo group during the study: BMI (kg/m²) (**A**), BMI z-score (kg/m²) (**B**) and waist circumference (WC) (cm) (**C**). "*" indicates $p<0.05$

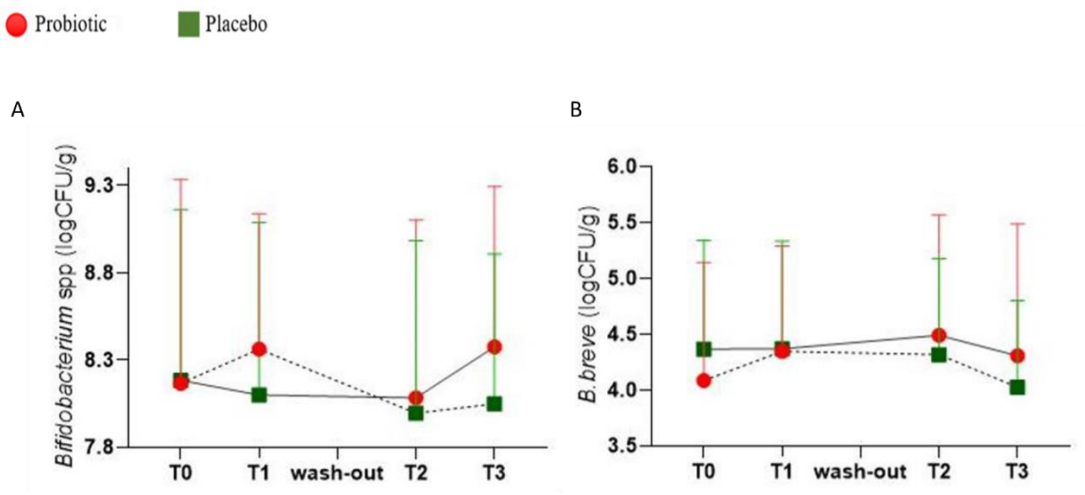

Fig. 4. Variations of *Bifidobacterium* spp. (logCFU/g faeces) (**A**) and *B. breve* (logCFU/g faeces) (**B**) in the probiotic and the placebo group during the study . "*" indicates $p<0.05$

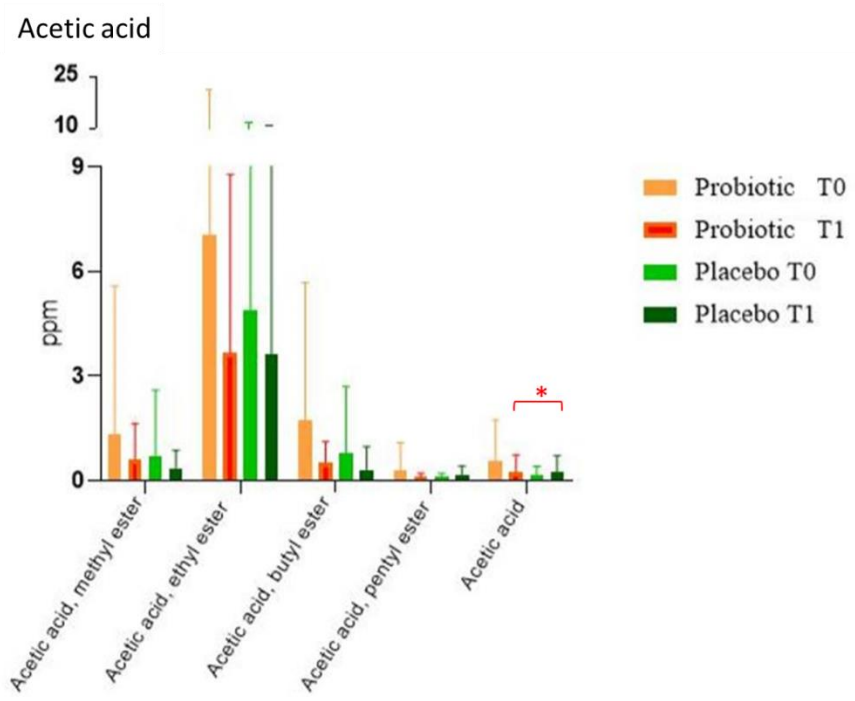

Fig. 5. Variations of acetic acid and its esters between the probiotic and the placebo group at T0 and T1. "*" indicates $p<0.05$

Clinical, metabolic and microbial characteristics in the phase 2 (T2-T3)

Compared to the time of recruitment (T0), the study showed at T2, after the wash out, significant changes in some parameters; in particular, reduction in BMI ($p < 0.01$) and BMI Z-score ($p < 0.05$), and increase in ISI ($p < 0.05$) and *Lactobacillus* counts ($p < 0.05$) were detected. In addition, for all the subjects, basal insulin ($p < 0.0001$) and HOMA-IR ($p < 0.01$) increased, while LDL cholesterol ($p < 0.0001$) (data not shown) and *E. coli* concentration were reduced ($p < 0.007$).
After the wash-out, significant differences were observed at the new baseline (T0 + T2) for the following parameters in the two groups: BMI ($p < 0.0001$), waist circumference ($p < 0.0001$), glycaemia 0' ($p < 0, 0001$), glycaemia 120' ($p < 0.0001$), insulin 0' ($p < 0.0001$), HOMA-IR ($p < 0.0001$), ISI ($p < 0.0001$), total cholesterol ($p < 0.0001$), HDL cholesterol ($p < 0.0001$), LDL cholesterol ($p < 0.0001$), triglycerides ($p < 0.0001$), IL-6 ($p < 0.0001$), IL-10 ($p < 0.0001$) and faecal concentration of *B. fragilis* ($p = 0.0038$) (data not shown). Analysing the trend over time of all subjects at phase 2, a significant reduction in BMI ($p < 0.0001$), waist circumference ($p < 0.02$), insulin 0' ($p < 0.05$) and an increase, not statistically significant, in HOMA-IR were observed. From T2 to T3 it was possible to observe a slightly decrease of BMI Z-score (**Fig. 3B**), an increase of glycaemia 0' (**Fig. 2D**) and a reduction of glycaemia 120'(**Fig. 2E**) in the probiotic group, while the placebo one remained quite stable for these parameters. Moreover, an increase of ISI only in the probiotic group was detected (**Fig. 2C**). *Bifidobacterium* spp. counts showed an increase in the probiotic group with respect to the placebo one, although not significant (**Fig. 4A**), while *B. breve* counts decreased in both groups (**Fig. 4B**). No significant differences in any of the other variables examined were observed between the probiotic and placebo group.

Table 4. Mean counts (Log CFU/g of faeces) of different microbial groups analyzed in stool samples of the whole cohort of subjects during the intervention.

Target	T0	T1	T2	T3
E. coli	6.40 ± 0.96	6.16 ± 1.11	6.27 ± 1.17	6.17 ± 1.17
***B. fragilis* group**	8.96 ± 0.93	8.93 ± 0.87	9.00 ± 0.89	9.02 ± 0.95
B. breve	4.28 ± 1.00	4.46 ± 0.86	4.56 ± 0.78	4.37 ± 0.99
***Bifidobacterium* spp.**	8.17 ± 1.08	8.18 ± 0.98	8.06 ± 1.00	8.27 ± 0.90
***Lactobacillus* spp.**	5.82 ± 1.04	5.72 ± 1.22	6.02 ± 1.12	5.68 ± 0.98

Table 5. Mean counts (Log CFU/g of faeces) of different microbial groups analyzed in stool samples of subjects during the intervention with probiotic or placebo.

Target	Probiotic T0	Placebo T0	Probiotic T1	Placebo T1	Probiotic T2	Placebo T2	Probiotic T3	Placebo T3
E. coli	6.09 ± 1.06	6.62 ± 0.84	6.04 ± 1.13	6.25 ± 1.10	6.37 ± 1.19	6.49 ± 1.16	5.95 ± 1.28	6.33± 1.07
***B. fragilis* group**	8.89 ± 0.84	9.07 ± 0.96	8.87 ± 0.92	8.98 ± 0.83	9.00 ± 1.07	9.03 ± 0.65	8.87 ± 1.00	9.14 ± 0.91
B. breve	4.09 ± 1.06	4.37 ± 0.97	4.49 ± 0.71	4.41 ± 0.99	4.53 ± 0.86	4.48 ± 0.66	4.46 ± 1.22	4.25 ± 0.63
***Bifidobacterium* spp.**	8.17 ± 1.17	8.18 ± 0.98	8.31 ± 0.82	8.08 ± 1.09	8.21 ± 0.99	7.89 ± 1.00	8.49 ± 0.86	7.99 ± 0.89
***Lactobacillus* spp.**	5,91 ± 0.87	5.72 ± 1.17	5.74 ± 1.48	5.70 ± 0.98	5.94 ± 1.16	6.07 ± 1.09	5.51 ± 0.95	5.82 ± 1.00

Discussion

According to the WHO, paediatric obesity represents one of the major public health challenges of the XXI century, whose prevalence has tripled over the last 40 years. For the onset and development of excess weight, to which genetic, environmental and behavioural factors contribute (Ang et al., 2013), an emerging role has been defined by the intestinal microbiota. In various experiments on animal and human models the positive effects of probiotic administration on BMI, adiposity, glyco-insulinemic and lipid metabolism and on the inflammatory response are documented (Amara and Shibl, 2015; Markowiak and Śliżewska, 2017). This study examines the effect of *B. breve* administration on obese children.

In obese subjects the microbiota is characterized by reduced levels of bifidobacteria (Ley et al., 2005, Kalliomaki et al., 2008). Moreover, the protective action on the microbiota balance, with consequent benefits to the host, has been documented especially for some strains of *Bifidobacterium* and *Lactobacillus* (Brusaferro et al., 2018). As bifidobacteria are present in abundance in the intestine of newborns, not showing any antibiotic-resistance, it was hypothesized that their annihilation by early antibiotic therapy in the first months or years of life could be one of the many factors implicated in the recent association of antibiotic therapy in the first two years of life to paediatric obesity (Saari et al., 2015). Furthermore, Mogna et al. (2012) highlighted the antagonistic ability of the two strains used in this study, *B. breve* BR03 and *B. breve* B632, against 4 pathogenic strains of *E. coli*. The intervention in our study was conducted for 8 weeks, according to some studies reviewed by Koutnikova et al. (2019) showing a potential colonization by probiotics administered for a period of the same duration of treatment.

Clinical, metabolic and microbial characteristics in the phase 1 (T0-T1)

Both in the probiotic and the placebo group at the end of the first 8 weeks there was an improvement of some biometric indices, with a reduction of BMI, BMI Z-score and waist circumference; probably, these effects are associated to the isocaloric dietary regime to which subjects adhered. In various experiments carried out on overweight and obese children and adolescents, it was evident that the nutritional intervention in association or not with physical

activity is correlated to a decrease of weight, body fat percentage and BMI (Santacruz et al., 2009; Miller et al. 1997; Garaulet et al., 2000; Sondike et al., 2003).

The probiotic administration evidenced a slight improvement in the auxological parameters, in accordance with several experiments that support the positive effects of bifidobacteria on body weight and visceral adiposity both in animal and human models (An et al., 2011; Minami et al., 2018; Stenman et al., 2016). However, the absence of statistical significance between the probiotic and the placebo group could depend on several factors, including the different genetic profiles, the age of the enrolled subjects, the dose of probiotic used, the duration of the treatment and the correct adherence to the associated diet therapy. Regarding the glyco-insulinemic metabolism, a reduction of insulin 0' and HOMA-IR was observed in both groups, suggesting the positive dietary contribution in improving insulin resistance. Similar positive effects are documented in individuals subjected to a nutritional intervention. The caloric restriction in several studies conducted on obese subjects with type 2 diabetes mellitus was correlated with a significant decrease in insulin resistance, increased β-pancreatic cell function, improved glycaemic control, decreased total cholesterol and blood pressure, with a consequent reduction of risk to develop cardiovascular disorders (Umphonsathien et al., 2019; Sellahewa et al., 2017; Norén and Forssell, 2014). An important outcome of the present study is that the probiotic administration led to an improvement in the insulin metabolism, particularly in HOMA-IR and ISI. The significant reduction of HOMA-IR is in agreement with a recent study that evidenced the positive effect on this parameter deriving from *B. animalis* subsp. *lactis* CECT81-45 supplementation in obese adults (Pedret et al 2018). The same effect, associated with an increase of ISI, was also found in mouse model with high fat diet-induced obesity administered with *B. breve* B03 (Kondo et al 2010). The significant increase of ISI observed in our study is in accordance with Rajkumar et al (2014), who highlighted the improvement of ISI in overweight adults supplemented with VSL#3 (Rajkusmar et al 2014).

Regarding the lipid profile, no significant differences were observed between the probiotic and the placebo group. The effect on lipid metabolism is controversial in the literature (Wang et al., 2018). The administration with *B. breve* B03 in murine models with diet-induced obesity and adults at risk of obesity was associated with an improvement of total cholesterol, triglycerides and HDL levels (Minami et al., 2018). In the study by Guardamagna et al. (2014), the administration in children with primary dyslipidaemia with *B. animalis* subspecies *lactis* MB2409, *B. bifidum* MB109B and *B. longum* subspecies *longum* BL04 for 3 months induced a significant reduction of triglycerides and LDL cholesterol. A significant improvement in total and LDL cholesterol has also been shown with the supplementation of *B. lactis* HN019 to 51 subjects suffering from metabolic syndrome (Bernini et al., 2015). However, the effect of probiotic administration on lipid metabolism reported in the literature is still to be explored. No alteration of lipid parameters was observed with the daily intake of a probiotic yogurt fermented with *B. lactis* and *L. acidophilus* (Ataie-Jafari et al., 2009; Ivey et al., 2015) and in association with *B. animalis* subsp. *lactis* BB-12 supplementation (Lee et al., 2007).

Regarding the effect on inflammatory cytokines, no significant differences between the probiotic and the placebo groups were found, thus suggesting, differently from other studies, that there was no effect of the probiotics on the inflammatory status. The two *B. breve* strains used in our study showed, instead, an anti-inflammatory effects with a significant decrease of TNF-α in the studies of Quagliariello et al. (2016) and Klemenak et al. (2015) conducted on celiac children. Similar results were also observed for other bifidobacteria, i.e. *B. lactis* HN01, *B. longum* subsp. *infantis* R0033

and *B. bifidum* R0071, whose administration to patients with metabolic syndrome induced a decrease of pro-inflammatory cytokines, leading to an improvement of the inflammatory profile (Ejtahed et al., 2011; Wang et al., 2007).
No significant differences in plasma LPS levels were found between the probiotic and the placebo group. In contrast, *in vitro* studies on intestinal epithelial cells HT-29 showed a dose-dependent capacity of some bifidobacteria, in particular *B. bifidum*, to exert anti-inflammatory effects inhibiting the NF-kB pathway activated by LPS (Riedel et al., 2006; Khokhlova et al., 2012). A negative correlation between bifidobacteria and the systemic toxicity induced by LPS has also been documented in various obese and/or diabetic murine models administered with *B. pseudocatenulatum* CECT 7765 (Moya-Pérez et al., 2015; Cano et al., 2013; Cani et al., 2007) and in rats with colitis after administration with *B. adolescentis* IM38 (Lim and Kim, 2017). However, considering that LPS may differ for structural characteristics and immunogenicity (Mogensen, 2009; Wang and Quinn, 2010), the two administered probiotics could have promoted the growth of bacteria not producing LPS or producing molecules that determined a reduced bacterial virulence and less susceptibility to activate the host innate immunity
With regard to the gut microbiota, beside the increase of *Bifidobacterium* spp. and *B. breve* counts due to the administration of the probiotic, no substantial alterations were found in the other analysed groups. This observation highlights the necessity of a deeper microbiological analysis, which is at present in progress, with the Next Generation Sequencing (NGS) technology.
From the evaluation of SCFAs, after 8 weeks of treatment, a significant reduction of acetic acid and two methyl esters of butyric acid (2-methylbutanoatodimethyl and 3-methylbutanoatimethylacetic acid) emerged in the probiotic group compared to placebo one. In the literature, a positive association between bifidobacteria and the levels of SCFAs has been highlighted, hypothesizing that the metabolites produced by bifidobacteria are used by other commensal microorganisms with consequent protective effects towards the intestinal barrier and the host (Belenguer et al, 2006; Rivière et al., 2016; De Vuyst and Leroy, 2011; Rivière et al., 2015; Falony et al., 2009a). Considering our study, however, it should be noted that the dispersion of the SCFAs was very high at the baseline, suggesting a high inter-individual variability in the gut environment of the study population. The NGS analysis of the gut microbiome in progress will allow a deeper analysis of the microbial composition biodiversity that can be correlated to the SCFAs results.

Clinical, metabolic and microbial characteristics in the phase 2 (T2-T3)

The study evidenced a significant difference of the clinical and metabolic characteristics of the study population after the 4-week wash-out period; it is possible to hypothesize that both the diet and the probiotic treatment contributed in a certain way to these differences, which persisted in spite of the wash-out.
The positive action of *B. breve* BR03 and *B. breve* B632 on body composition and glyco-insulinemic metabolism, documented after the first 8 weeks of treatment, was not observed in the second phase of the study. Therefore, it is possible to hypothesize that the benefits on paediatric obesity of the 2 bifidobacteria species used occur at a certain state of the disease. However, a possible contribution of the diet to the failure to find positive effects in this second phase of the study cannot be excluded. At the time of recruitment an isocaloric diet was recommended, but the adherence to it was not assessed through a specific questionnaire.

Concerning the microbial gut composition, no significant changes were detected; the high data scattering observed between the two groups probably hampers the statistical significance output. It is possible to speculate that the wide interpersonal diversity of the microbial profiles may determine the different response capacity to both probiotic treatment and dietary intervention. The intestinal microbiota composition tends to fluctuate especially during childhood, being influenced by multiple factors (diet, antibiotics, psycho-physical stress, integrity of the immune system and digestive functions) and remains relatively stable in physiological conditions in adulthood (Falony et al., 2016). Moreover, it is possible that a probiotic treatment of 8 weeks did not impact enough on gut microbiota of this kind of patients, therefore a prolonged intervention should be considered, as reported in other studies conducted on obese individuals (Minami et al., 2015; Minami et al., 2018, Stenman et al., 2016). Again, the metagenomic analysis with NGS will allow a better understanding of the microbiota population composition and the differences between the two groups, as well as the possible effects of the combined nutritional and probiotic intervention on the microbial groups that have not been analysed with qPCR in the study. In addition, the analysis of SCFs in the phase 2 of the study, which is ongoing, will provide further information about the effectiveness of the probiotic intervention.

Conclusions

The treatment with *B. breve* BR03 and *B. breve* B632 in obese children and adolescents on a diet therapy determined a significantly positive effect on glyco-insulinemic parameters, whereas the influence on the body composition and gut microbiota, although present, was not significant. Metagenomic studies, which are ongoing, will allow us to characterize the whole gut microbiota of the subjects in the study, providing further information on the ability to respond to both probiotic treatment and nutritional intervention and additional correlations with clinical parameters.

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Paper 6

A prospective longitudinal study on the microbiota composition in Amyotrophic Lateral Sclerosis

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This paper is dedicated to Dr. Giovanni Mogna, who strongly believed in this work.

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Abstract

Background. A connection between Amyotrophic Lateral Sclerosis (ALS) and altered gut microbiota composition has previously been reported in animal models. This work is the first prospective longitudinal study addressing the microbiota composition in ALS patients and the impact of a probiotic supplementation on the gut microbiota and disease progression. **Methods.** Fifty patients and 50 matched healthy controls were enrolled. The microbial profile of stool samples from patients and controls was analyzed via PCR-Denaturing Gradient Gel Electrophoresis and the main microbial groups quantified via qPCR. The whole microbiota was then analyzed via Next Generation Sequencing after amplification of the V3-V4 region of 16S rDNA.
Results. The results demonstrate that the disease is associated to a gut microbial composition intrinsic to the disease. Moreover, the gut microbiota changes during the course of the disease and can influence its progression. Some microbial groups may facilitate the pathogenesis of the disease such as Cyanobacteria, potentially influencing inflammatory cytokines and bacterial metabolites production, while others may provide neuroprotection against the progression of the disease. The 6-months probiotic treatment influenced the gut microbial composition; however, it did not bring the biodiversity of intestinal microbiota of patients closer to that of healthy subjects and influenced the progression of the disease.
Conclusions. Our study poses the bases for larger clinical studies to characterize the microbiota changes as a novel ALS biomarker and to test new microbial strategy to ameliorate the health status of the gut.

Trial registration: CE 107/14, approved by the Ethic Committee of the “Maggiore della Carità” University Hospital, Italy.

Key words: Amyotrophic Lateral Sclerosis, neurodegeneration, biomarker, microbiota

Background

Amyotrophic Lateral Sclerosis (ALS) is a devastating, incurable neurodegenerative disease that affects the upper and the lower motor neurons leading to death by respiratory failure within 2-5 years from the onset of the disease. The etiology is still unknown and the pathogenesis remains unclear. ALS is familial in the 10% of cases with a Mendelian pattern of inheritance while in the remaining sporadic cases a multifactorial origin is supposed in which several predisposing genes interact with environmental factors in manifesting the disease [1]. Imbalance in the gut microbiota composition may be one of the environmental factors contributing to the development of ALS. The composition of the intestinal microbiota is gaining importance in human health studies since there is increasing evidence that its alteration plays a role in disease etiology. The gut microbiota represents an important boundary between the environment and the immune system, and a major site for exposure to a wide range of both pathologic and intrinsic antigen and toxin production. It has been hypothesized that the intestinal microbiota can represent an epigenetic entity that interacts with environmental factors in determining pathogenic influence also on the Central Nervous System (CNS) [2].

Emerging evidences link alterations of the gut microbiota to the risk and the severity of some neurodegenerative diseases, such as in Parkinson's disease (PD), where patients showed a lower abundance of Prevotellaceae members with respect to healthy controls [3] and a correlation between specific taxa and different motor phenotypes [3,4], and in Alzheimer's disease (AD), finding differences in some microbial groups (e.g. Actinobacteria*,* Lachnospiraceae*, Rominococcus, Bacteroides*) compared to controls [5].

Several studies have hypothesized a role of the gut microbiota in the alteration of circulating levels of inflammatory cytokines or in the production of neurotoxins, which are known to affect the CNS and may have a role in the development or progress of neurological disorders [3,6–9]. A correlation of ALS with altered gut microbiota composition has previously been reported in animal models [10,11] while only few preliminary studies have analyzed the composition of the fecal microbiota in ALS patients with no conclusive results [12,13]. Rowin et al. [13] showed, on a restricted number of patients, a lower Firmicutes/Bacteroidetes (F/B) ratio, used as a marker of intestinal disbyosis, as well as a lower *Ruminococcus spp* abundance in ALS patients with respect to controls. Brenner et al. [12], in 25 ALS patients, observed a higher OTU richness in ALS patients with respect to healthy controls, without significant differences in biodiversity indices.

Methods

This work is a prospective longitudinal study addressing the microbiota composition in ALS patients and matched controls with the aim to consider the possible impact of a probiotic supplementation on the gut microbiota and disease progression.

Study design

This study was primarily designed as a prospective longitudinal study to evaluate the microbiota composition in a population of ALS patients compared with a case-control group of unrelated subjects matched for sex, age, origin and eating habits (unrelated members of the family or friends). The patients were then randomized, in a double blinded, placebo-controlled, monocentric trial to

receive a supplement or placebo in order to verify the changes of the microbiota composition with respect to the progression of the disease and the effects of the probiotic supplementation.
The study has been approved by the Ethic Committee of the "Maggiore della Carità" University Hospital (CE 107/14). All participants provided a written informed consent. The patients were enrolled at the Tertiary ALS Center in Novara in a period from January 2016 and September 2017.
We enrolled 50 sporadic ALS patients with a diagnosis of probable or defined ALS according to El Escorial Criteria [14], aged 18 to 75 years, within 3 years from diagnosis, and Force Vital Capacity percentage (FVC%) > 50%. We excluded patients with percutaneous endoscopic gastrostomy or nasogastric tube, tracheotomy or non-invasive ventilation for more than 18 hours/day, unable to understand informed consent.
We also excluded patients and controls with concomitant diseases (i.e. malignant neoplasms, gastrointestinal, inflammatory, autoimmune, cardiovascular and respiratory disease) and subjects who have taken drugs or antibiotics that may modify the intestinal microbiota in the 8 weeks prior to recruitment. Patients received continuous riluzole treatment (100mg/day), and symptomatic treatments. All patients were screened for the presence of mutations in the most common gene related to ALS (SOD1, C9Orf72, TARDP, FUS). All patients, at the baseline, underwent clinical and neurological evaluation that included the compilation of the ALS Functional Rating Scale – Revised (ALSFRS-R) score, spirometry with the measurement of FVC%, collection of Body Mass Indices (BMI) with a dietary assessment to know the eating habits, the impairment of the autonomous feeding, chewing, swallowing; the weight loss compared to pre-morbid weight.
After one observation month, patients were randomized to double-blind treatment either to the supplement or to placebo. The first group received a probiotic-based formulation for six months (Group A) and the second one an equal dose of placebo for three months and then the probiotic-based formulation for the other three months (Group B). Statistic unit will assign unique treatment code for all patients. Subjects, investigators, clinical and laboratory staff were blinded to treatment group assignment.
The follow-up considered monthly monitoring for six months. At each visit, the disease severity was assessed with the ALSFRS-R score, pulmonary function tests to calculate the FVC%, and a nutritionist calculation of BMI.
Stool samples were collected from ALS patients at the baseline (T0), after 3 months (T1) and 6 months (T2) and from healthy controls (H) at the baseline.
After DNA extraction from stool samples, DNA from patients and controls at T0 was analyzed via PCR- Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis. Selected microbial groups from patients (T0, T1, T2) and controls (T0) were quantified via qPCR and the whole microbial community were analyzed from the same samples via Next Generation Sequencing (NGS) approach after amplification of the V3-V4 region of 16S rDNA.

Probiotic Supplement

The probiotic formulation is a mixture of five lactic acid bacteria administered in the following daily dosages: *Streptococcus thermophilus* ST10 - DSM 25246, 5 x 10^9 CFU/dose; *Lactobacillus fermentum* LF10 – DSM 19187, 4 x 10^9 CFU/dose; *Lactobacillus delbrueckii* subsp. *delbrueckii* LDD01 – DSM 22106, *Lactobacillus plantarum* LP01 – LMG P-21021, *Lactobacillus salivarius* LS03 – DSM 22776, 2 x 10^9 CFU/strain/dose. This probiotic formulation is ad-hoc designed, patented and produced by Probiotical SPA – Novara, Italy. The choice of the strains was done

considering the results of previous studies that showed their capabilities to counteract gut pathogens, their anti-inflammatory properties and positive influence in restoring the gut physiological barrier [15–18].

DNA extraction from fecal samples

Stool samples were stored at -80 °C until analysis. Total genomic DNA was extracted by using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer 's instruction with a slight modification of the standard protocol according to Aloisio et al. [19] and an additional treatment with lyticase (Sigma-Aldrich, Milan, Italy) at 37 °C for 30 min. The purity of extracted DNA was evaluated measuring the ratio of absorbance at 260 and 280 nm (Infinite®200 PRO NanoQuant, Mannedorf, Switzerland) and the DNA concentration estimated with the Qubit® 3.0 Fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, USA).

Absolute quantification of selected microbial groups using quantitative PCR (qPCR)

Absolute quantification of *Lactobacillus* spp, *Bifidobacterium* spp, *Clostridium* cluster I (including *C. baratii*, *C. hystoliticum*, *C. butyricum*, *C. prefringens*, *C. botulinum* and *C. tetani*), *Escherichia coli*, Enterobacteriaceae and total yeasts was performed with qPCR using the Fast SYBR®Green Master Mix (Applied Biosystems, Foster City, USA) and optimized concentrations of primers [20–23]. Standard curves were constructed using 16S rRNA PCR product of type strains of each target microorganism [23,24] and data transformed to obtain the number of microorganism as Log CFU/g feces according to the rRNA copy number [25]. For total bacteria, the average of the 16S rRNA genes calculated on 10996 records for Bacteria according to rrnDB was used as the rRNA copy number [26,27].

For yeasts, not being available the rRNA copy number, a normalization of the number of yeast cells was performed, before the conversion in Log CFU/g feces.

PCR-DGGE

PCR-DGGE analysis was performed before the enrollment was completed on the first 38 healthy and 38 diseased subjects recruited in order to have a preliminary investigation of total eubacteria and yeast populations. DNA was amplified using primers targeting the V2-V3 region of 16S rDNA and the D1 region of 26S rDNA, for Eubacteria and yeasts, respectively [28,29]. DGGE analysis on obtained amplicons was performed as described previously [28,30], using the Dcode System apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Patterns were normalized by including a ladder with PCR products obtained from known pure cultures. Similarities and a cluster analysis among DGGE profiles were carried out using the Gel compare II v6.6 (Applied Maths, St. Martens-Latem, Belgium), by the unweighted pair-group method with the arithmetic average (UPGMA) clustering algorithm based on the Dice coefficient with an optimization coefficient of 1%.

Preparation of DNA Libraries for Illumina MiSeq Sequencing

DNA samples were subjected to Illumina sequencing. The V3-V4 region of the 16S rRNA gene was amplified and sequenced. One sample was excluded as it did not pass the established quality threshold. The amplicons, approximately 460 bp in length, were generated using the forward and reverse primers, respectively: 5'-CCTACGGGNBGCASCAG-3' and 5'-GACTACNVGGGTATCAATCC-3' [31]. The assays were performed using a previously published

protocol with some modifications [23]. The sequencing process was outsourced at Macrogen Inc. (Next Generation Sequencing Division), Seul, Republic of Korea, using a 2x300 pair-end protocol.

Bioinformatic and statistical analysis

Resulting 300 bp paired-end reads were assembled using FLASH [32]. Further sequence read processing was performed using QIIME ver. 1.9.1 [33] and ChimeraSlayer [34], including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH version 7 [34]. OTU sequences were aligned using PyNAST [35] and taxonomy assignment was determined using the SILVA SSU Ref database release 111 [36]. Biodiversity indices analysis was performed using QIIME tools, in particular the script "core_diversity_analysis.py"; the phylogenetic classification of OTUs was carried out with the script "make_phylogeny.py" (fasttree). α-diversity was evaluated considering Chao, Observed OTU and PD whole tree metrics; β–diversity was evaluated using 'weighted_unifrac' method [37].

For α–diversity indices comparison, the normality and the homogeneity of variance of datasets were checked; statistical significance was evaluated with one-way ANOVA; two-way repeated measure ANOVA comparing different variance-covariance models was used to evaluate time-treatment interactions. Non-normal and non-homoscedastic datasets were compared with Kruskall-Wallis test.

For β–diversity indices comparison, data resulting from QIIME statistical elaboration were reported; the software performed 100 randomizations of sample/sequence assignments, and recorded the probability that sample 1 is phylogenetically different from sample 2, using the unifrac Monte Carlo significance test. The test was run for all pairs of samples. The *P*-value was adjusted according to the Bonferroni correction for the comparisons performed.

F/B ratio values were calculated as indicator of dysbiosis, for each group of subjects [38].

Data of microbial counts were subjected to T-student test to evidence significant differences between ALS patients and healthy control at baseline, and between treated and control group during the study period.

Results

Subjects

Between January 2016 and September 2017 400 patients with ALS were screened at the tertiary ALS Centre in Novara, Italy. Fifty patients (28 males) and 50 matched healthy controls (28 males) were enrolled. The mean age at entry was 60.24 (standard deviation, SD 10.76) in patients and 53.60 (SD 15.34) in the control group.

After one-month observation period ALS patients were randomly assigned to receive either placebo (n=25) or probiotics (n=25). The demographic and clinical profiles of the two groups at entry are shown in Table 1 and were comparable. The clinical features have remained comparable between Group A and Group B also at T1 and T2.

Two patients enrolled in the study and without a family history for ALS or Frontotemporal Dementia showed the GGGGCC hexanucleotide expansion in the first intron of *C9orf72*.

One patient died for the progression of the disease. 20 patients discontinued the study before the conclusion of their six months follow-up period and were documented as dropouts.

Table 1 Clinical features of ALS patients at baseline for Group A and Group B

Clinical features	Group A	Group B
Number (sex)	25 (M 15)	25 (M 13)
Age (SD)	60.36 (10.86)	59.12 (10.86)
Weight (kg, SD)	70.10 (13.38)	64.89 (13.28)
BMI (SD)	24.82 (3.95)	22.12 (5.17)
Spinal onset (n, %)	20 (80%)	22 (85%)
Disease duration (months, SD)	26.32 (24.97)	20.69 (18.75)
FVC% (SD)	81.48 (18.28)	84.15 (18.44)
ALSFRS-R (SD)	36.56 (5.82)	35.81 (5.81)

The values are expressed as mean (SD). BMI, body mass index; FVC, forced vital capacity; ALSFRS-R, ALS functional rating scale revised. *P*-value: not statistically significant for each analysis.

Baseline characteristics

PCR-DGGE

The cluster analysis of the 16S rDNA generated by DGGE using the UPGMA algorithm is shown in Fig. 1a. The fingerprint of the intestinal Eubacteria was characterized for each subject by 30-40 detectable bands, which differed in number, position and intensity. Except for 7 profiles (5 diseased and 2 healthy) forming a unique group with similarity lower than 59% (bottom part of Fig. 1a), two major clusters were obtained: one grouping 17 healthy subjects (similarity ~ 60.6%, Group G1 in Fig. 1a) and one larger containing 3 sub-clusters: the first one (Group G2) composed of 12 healthy subjects, the second one including 20 diseased samples (Group G3), i.e. more than 50% of the total diseased patients considered in the analysis, and 2 healthy profiles (similarity less than 65.2%) and the third one containing 14 diseased samples and 5 healthy ones.

Yeast profiles, obtained from the cluster analysis of the amplified D1 region of 26S rDNA, are much simpler than those of Eubacteria (Fig. 1b). Overall, no unequivocal grouping of diseased/healthy profiles could be observed. The dendrogram obtained from the DGGE banding patterns showed two distinct sub-clusters, according to the number of bands as well as their positions, each composed of both healthy and diseased subjects.

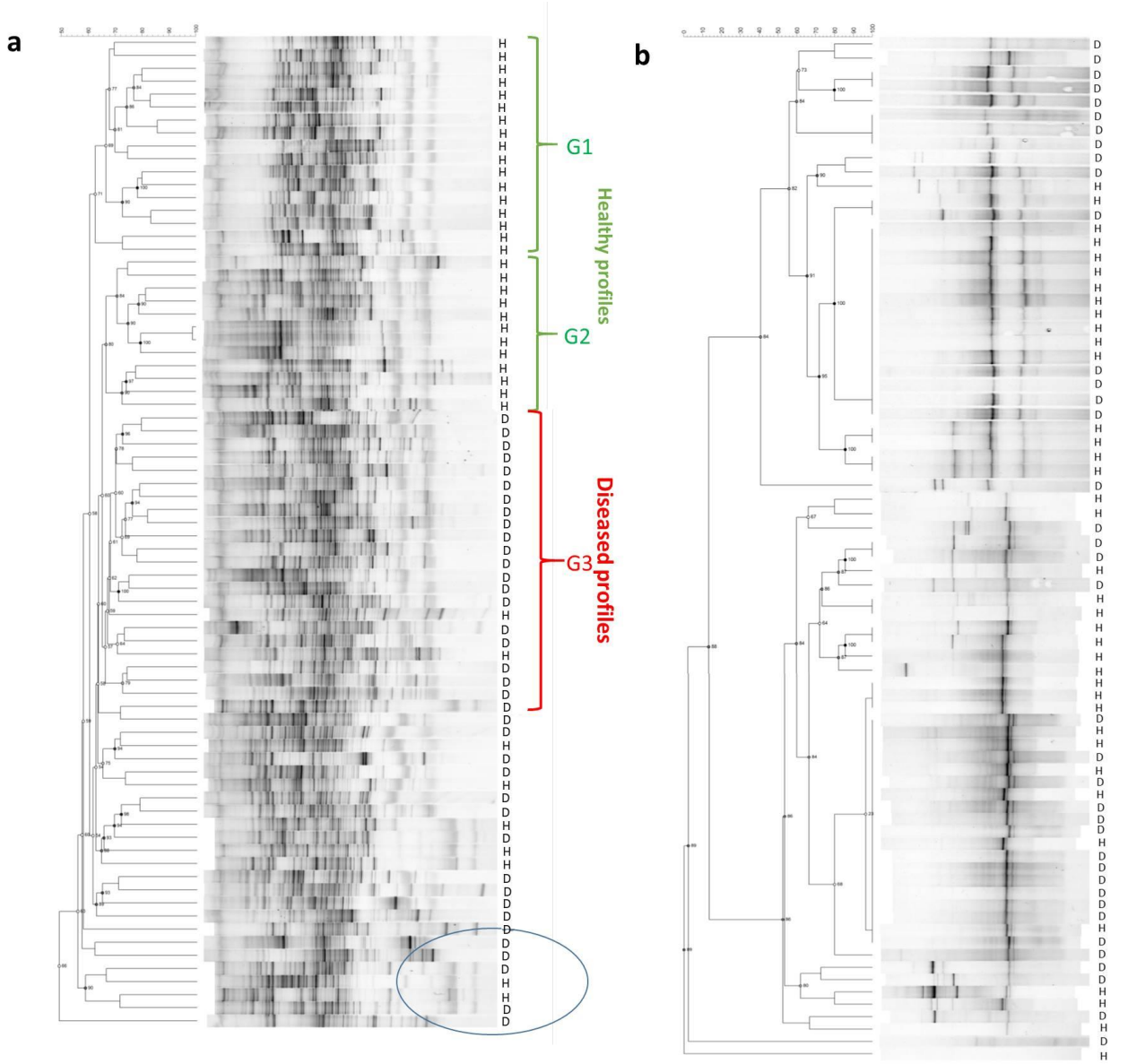

Fig. 1 Gut bacteria and yeasts profiles at baseline obtained by DGGE. **a** UPGMA dendrogram and DGGE profiles of eubacteria. Three main cluster groups are indicated as G1, G2 and G3: G1 and G2 represent healthy subjects (H) and G3 represents diseased subjects (D). **b** UPGMA dendrogram and DGGE profiles of total yeasts in healthy (H) and diseased (D) subjects.

qPCR

DNA quantification after extraction from the same amount of stool showed, at the baseline, a lower DNA concentration in patients compared with controls (Table 2). However, the number of total bacteria was not significantly different in the two groups. A significant lower amount of *Clostridium* cluster I and yeasts and a significantly higher concentration of *E. coli* were detected in ALS patients with respect to controls; Enterobacteriaceae were higher in ALS subjects (P=0.05). No significant differences were found in *Bifidobacterium* and *Lactobacillus* spp (Table 2).

In addition, higher values of FVC% and ALSFRS-R significantly correlated with a greater amount of yeasts in the microbiota (P<0.001 and 0.03, respectively). No significant correlations were found with the BMI.

Table 2 DNA concentration and mean counts of the analyzed microbial groups by qPCR in stool samples of ALS patients (case) and healthy controls.

	Sample	Mean (SD)	*P*-value
DNA	case	155.50 (118.68)	**0.02**
	control	210.10 (97.25)	
Total bacteria	case	10.36 (0.86)	0.90
	control	10.34 (0.74)	
Lactobacillus spp.	case	5.44 (1.26)	0.09
	control	5.77 (0.76)	
Bifidobacterium spp.	case	7.30 (1.63)	0.54
	control	7.43 (1.28)	
E. coli	case	6.60 (1.13)	**0.04**
	control	6.0 (1.61)	
Clostridium cluster I	case	5.72 (1.55)	**0.01**
	control	6.39 (0.84)	
Enterobacteriaceae	case	8.51 (0.8)	**0.05**
	control	7.96 (1.84)	
Total yeast	case	5.78 (0.81)	**0.02**
	control	6.07 (0.65)	

The DNA concentration is expressed as ng/200 mg of faeces and the mean counts as Log CFU/g of faeces, the related *P*-value is reported. Bolded values indicate $P \leq 0.05$.

NGS analysis

A total dataset of 13,592,139 filtered high-quality joined reads was generated after sequencing the V3-V4 region of 134 DNA samples, obtaining about 101,433 sequences per sample, with a mean quality in the range 30-38. Fig. 2a and 2b show the major phyla belonging to Bacteria and Archaea in healthy and diseased subjects. The more representative phyla were Bacteroidetes and Firmicutes in both groups, which showed a relative abundance of 40-45%; a higher percentage of Actinobacteria and Verrucomicrobia was detected in the diseased group (3.5 and 6.6 %, respectively) compared to the healthy one (2.6 and 3.6%, respectively). Members of the Cyanobacteria phylum were significantly higher ($P<0.05$) in the diseased group with respect to the control one (0.3% *vs* 0.2%, respectively) (Fig. 2c).

In addition, the heat map presented in Additional file 1: Figure S1a shows several differences at the family level between the two groups. Some families presented a higher relative abundance in the diseased group, even though not of statistical significance: Clostridiales vadinBB60 group, belonging to Clostridia, Bacteroidales S24-7 group, Coriobacteriaceae, Verrucomicrobioaceae and Lactobacillaceae. On the other hand, the healthy group showed a higher relative abundance of Veillonellaceae, Promicromonosporaceae and Peptostreptococcaceae.

The difference in the Cyanobacteria phylum is reflected at the family level in the significantly higher abundance in ALS patients of Gastranaerophilales (uncultured bacteria) (P<0.05). A significantly different amount was also found in families related to Clostridiaceae, such as Clostridiales Ambiguous taxa and Clostridiaceae 1, which were lower in patients (P<0.05) whereas Clostridiales Family XI resulted higher in patients (P <0.05) (Fig. 2d).

At the genus level (Additional file 1: Figure S1b), *Lactobacillus*, *Citrobacter*, *Coprococcus* and some genera belonging to Ruminococcaceae (including *Ruminiclostridium*) were found to be more adundant in patients. In addition, genera belonging to Enterobacteriaceae (such as *Escherichia* and *Shigella)*, *Akkermansia*, *Eubacterium eligens* group, *Odoribacter*, *Bifidobacterium, Pseudoflavonifractor* and other genera belonging to Prevotellaceae and Ruminococcaceae, specifically Ruminococcaceae NK4A214 group and Ruminococcaceae UCG-014, manually annotated as *Intestinimonas* and members of the family Hungateiclostridiaceae, respectively, were also more abundant in ALS patients. Genera belonging to Veillonellaceae and Lachnospiraceae (Lachnospiraceae_Eubacterium) families, the genus *Parasutterella*, *Ruminococcus* and *Subdogranulum*, both belonging to Ruminococacceae, were, on the contrary, more abundant in the healthy control group. Two genera belonging to Gastranaerophilales were more abundant in diseased subjects (P<0.05); all the other differences at the genus level were not statistically significant (P=0.05 for *Ruminiclostridium*).

Differences in the microbial community biodiversity between healthy and diseased subjects were observed by calculating the value of α- and β-diversity. The Chao1 index (α-diversity), related to the abundance of sequences for each OTU, was significantly higher (P <0.05) in the healthy control group with respect to diseased subjects (Fig. 2e). The other α-diversity indices, observed OTU and PD whole tree, were not significantly different between the two groups. Significant differences (P <0.05) in the Chao1 index were also detected subgrouping ALS patients for their ALSFRS-R scale. Score. Patients were classified in High Functional Rating Scale (HFRS) with score ≥ 35 and Low Functional Rating Scale (LFRS) < 35. The statistical analysis showed significant differences between the group of healthy and LFRS patients (Fig. 2f). β-diversity was also significantly different (P<0.05) between healthy controls and ALS patients, even if the PCoA analysis didn't show a clear division between the two groups (Fig. 2g); however, a scattered trend for each individual, especially for healthy subjects, was depicted in the PCoA analysis (Fig. 2g). Statistically significant differences in β-diversity resulted between healthy subjects and HFRS group (P <0.005). Moreover, within the diseased group at the baseline differences were observed between HFRS and LFRS (P<0.05), particularly *Eubacterium eligens* group was more abundant in HFRS with respect to LFRS. Patients with C9Orf72 expansion did not differ in all examined parameters.

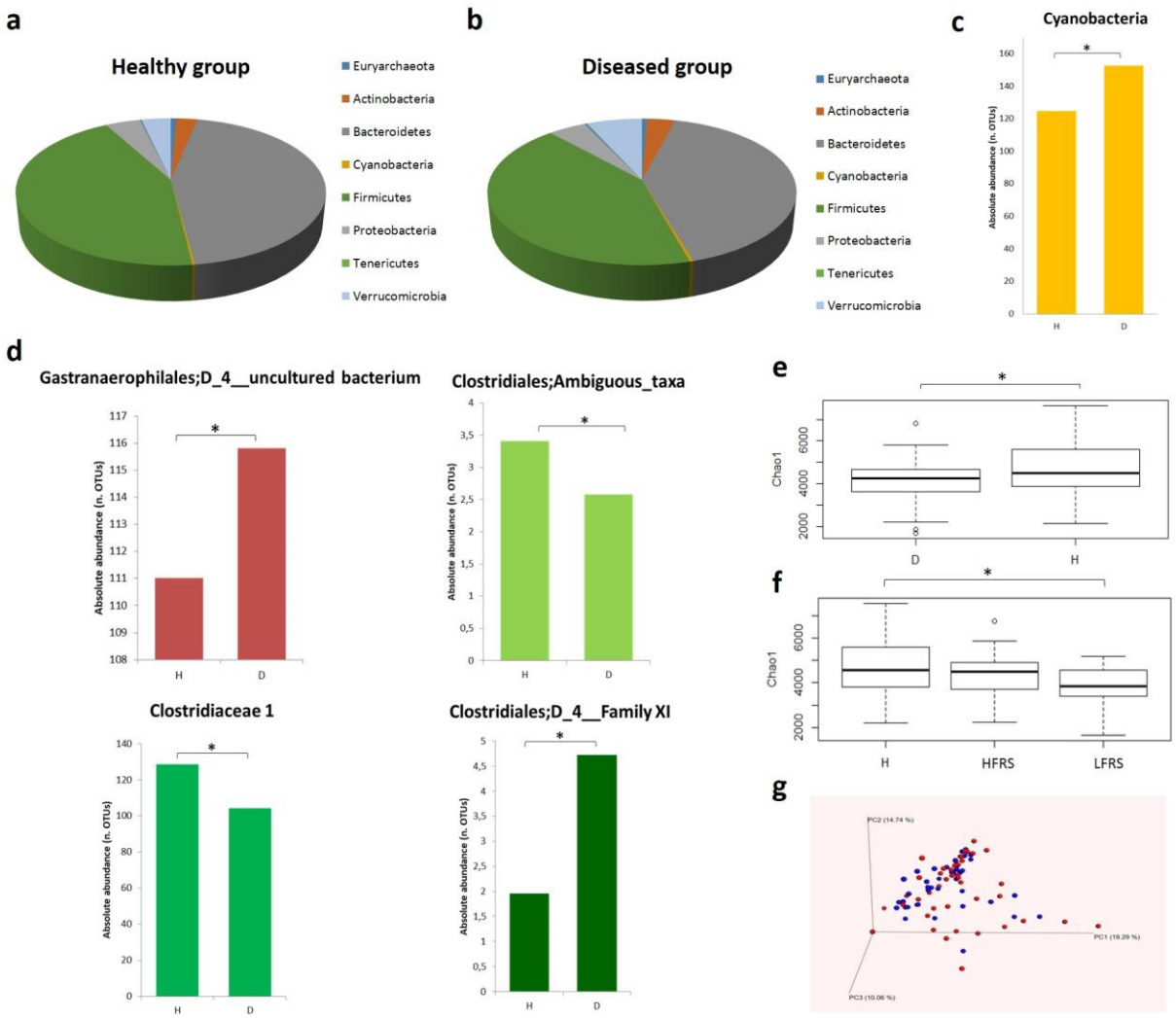

Fig. 2 Gut bacterial characteristics at the baseline and main differences between healthy and diseased subjects. **a** Relative abundance of the main bacterial phyla in healthy group. **b** Relative abundance of the main bacterial phyla in diseased group. **c** Differences (P<0.05) in Cyanobacteria between healthy (H) and diseased (D) subjects; data are expressed as absolute abundance (number of OTUs). **d** Bacterial groups classified at family level showing significant differences (P<0.05) between healthy (H) and diseased (D) subjects. **e** Differences (P<0.05) in α-diversity Chao1 index between diseased (D) and healthy subjects (H). **f** Differences (P<0.05) in α-diversity Chao1 index in ALS patients subgrouped for their ALSFRS-R scale values (HFRS ≥ 35, LFRS < 35). **g** PCoA representing β-diversity among individuals at baseline (healthy subjects=red, ALS subjects=blue).

Probiotic/placebo supplementation in ALS subjects

qPCR

No Adverse Events (AEs) attributed to probiotic supplementation and no AEs of special concern, such as diarrhea or gastrointestinal symptoms occurred.

The same microbial groups analyzed at the baseline (T0) were quantified at T1 (3 months) and T2 (6 months), (Additional file 1: Table S1). No significant differences were observed in total bacteria counts, although at T1 a lower bacterial counts were observed in Group B with respect to Group A. DNA concentration extracted from 200 mg of faecal material decreased, although not significantly,

with the progression of the disease. A significant reduction of yeast concentration in T2 Group A with respect to T2 Group B (P=0.03, Additional file 1: Table S1 and Figure S2) was found. No significant differences were observed for the single bacterial groups, except for an increase (P=0.05) of *E. coli* in Group B patients with respect to the group of patients (Group) A that received the probiotic for 6 months. No significance was also observed by correcting the data for the time/treatment interaction. Observed changes in ALSFRS-R, FVC (%) and BMI did not differ between the two groups.

NGS analysis

Fig. 3 shows the relative abundance at the phylum level of each ALS patient before and after each treatment at the different times. A large variability among individuals was detected. The abundance of Cyanobacteria decreased over time in both probiotic and placebo group, although not significantly. Euryarchaeota and Actinobacteria were more represented in the group of patients that received the probiotic formulation after an initial treatment with placebo (T2 Group B) with respect to the other groups. Moreover, some individuals presented higher levels of Synergistetes members (2-5%) with respect to the baseline independently of the treatment.

Fig. 3 Relative abundance of gut microbial phyla in ALS patients during the intervention. Group A consists of subjects receiving the probiotic treatment for 6 months (T0=baseline, T1=3 months from baseline, T2=6 months from baseline). Group B consists of subjects receiving the placebo treatment for 3 months (T1) and the probiotic treatment for the following 3 months (T2). Phyla with a percentage of relative abundance less than 0.002% are grouped in "other".

At the phylum level, considering time as the only variable not including the different type of treatment, the analysis showed a significant decrease of one of the less abundant phyla, Tenericutes, and a significant increase of one of the major phyla, Bacteroidetes, at T1 with respect to T0 (Fig. 4a and 4b). The phylum of Spirochaetae was present only in two ALS patients, which received the placebo formulation followed by the probiotic one, and showed an increase in the number of reads over time.

All groups of individuals were compared considering the Firmicutes/Bacteroidetes ratio (Fig. 4c). No statistically significant differences were obtained in the F/B values, although the value was the highest in T2 Group B.

At the family level, the most represented families were Bacteroidaceae, followed by Ruminococcaceae, Lachnospiraceae and Rikenellaceae (Additional file 1: Table S2). Bifidobacteriaceae transitorily increased after 3 months of probiotic treatment in both ALS patient groups, although not significantly. Bacteroidaceae increased in all patients, but particularly in the Group A (Fig. 4d). Bacteroidaceae, together with other families belonging to Bacteroidales, significantly changed over time, not considering the type of treatment (Fig. 4e and 4f). A significant increase in Rikenellaceae relative abundance was associated to six-months probiotic treatment (Fig. 4g); moreover, Group A T2 had considerable higher levels of Rikenellaceae (12.25%) with respect to Group B T2 (4.82%) (Additional file 1: Table S2). A relevant drop of Prevotellaceae, Christensenellaceae and Clostridiales vadinBB60 group was detected in Group A T2. The family of Clostridiaceae 1 decreased in both groups after six months from baseline, thus increasing the difference from the value associated to healthy subjects (Additional file 1: Table S2). Lachnospiraceae only diminished in Group A. Ruminococcaceae showed the same trend in the two ALS groups, decreasing at T1 and then increasing at T2. Regarding Veillonellaceae, a stronger reduction was detected in Group B at T1; in any case the abundance remained higher in controls. Interestingly, the Verrucomicrobiaceae levels, resulting higher in ALS patients.

Regarding α-diversity indices, a significant decrease was reported in the number of observed OTU in ALS patients between three (T1) and six months (T2) from baseline, not considering the type of supplementation but only time as a variable (Fig. 4h). The number of observed OTU was also significantly lower in ALS subjects with a spinal onset at six months from baseline (T2), compared to healthy controls (Fig. 4i). No differences were observed for Chao1 and PD whole tree indices. The β-diversity analysis showed significant differences in Group A at T1 and T2 with respect to the baseline. Patients subjected to three-months probiotic treatment (T2 Group B) showed significant differences compared to the previous placebo treatment (T1 Group B). Moreover, differences were observed between the group receiving the probiotic for 3 months (T2 Group B) and the group receiving the probiotic for 6 months (T2 Group A) (Table 3). The β-diversity was not significantly different subgrouping patients for ALSFRS-R score; however, the healthy group was significantly different both from the HFRS and LFRS groups at the baseline ($P<0.05$).

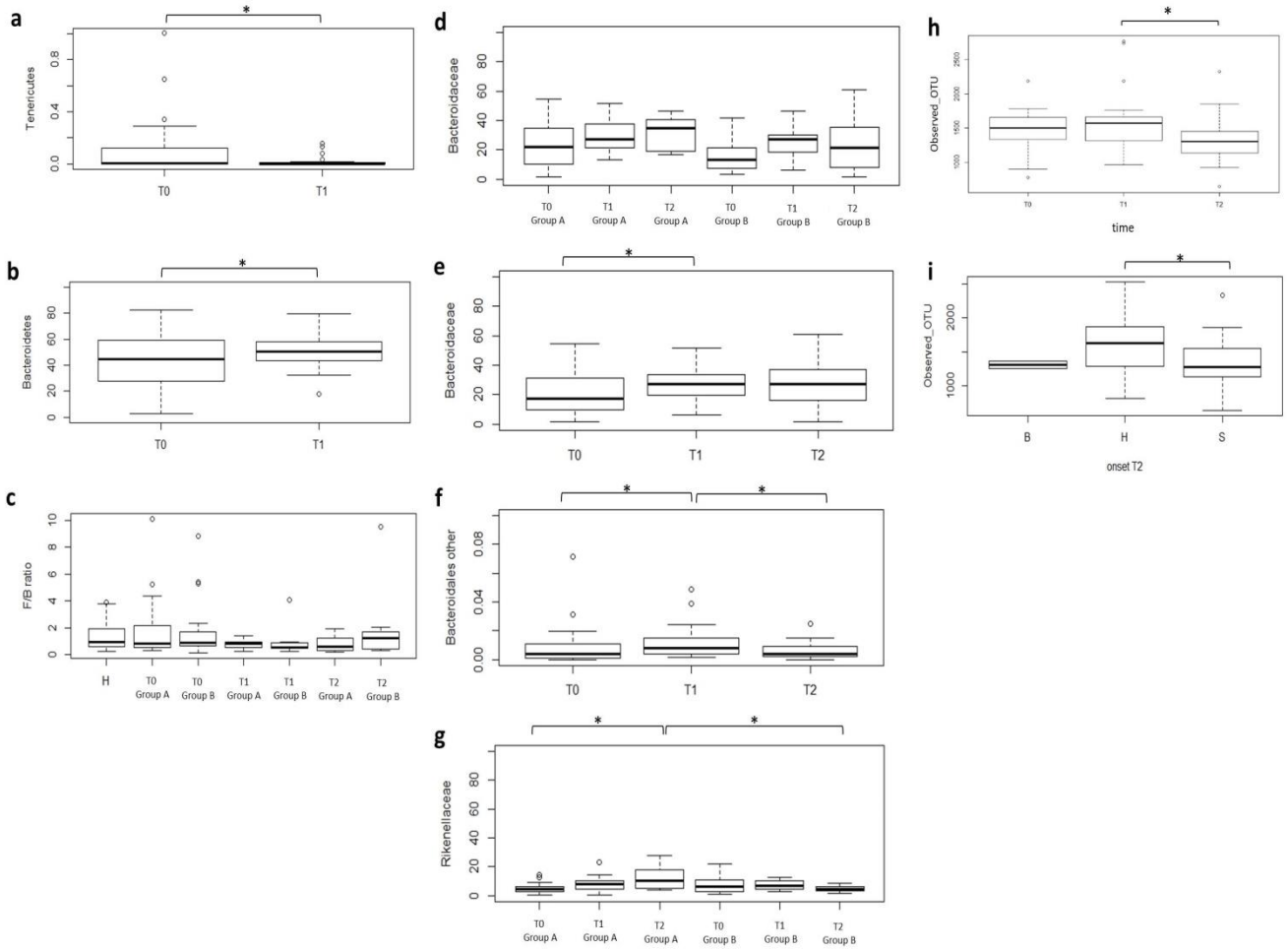

Fig. 4 Gut bacterial characteristics and main differences within the study groups during the intervention**. a** Differences (P<0.05) in Tenericutes relative abundance after 3 months (T1), not considering the type of treatment. **b** Differences (P<0.05) in Bacteroidetes relative abundance after 3 months (T1), not considering the type of treatment. **c** F/B ratio in the different time-treatment groups. **d** Relative abundance of Bacteroidaceae in ALS patients considering the time and the type of treatment. **e** Differences (P<0.05) in Bacteroidaceae relative abundance within time, not considering the type of treatment. **f** Differences (P<0.05) in relative abundance of other families belonging to Bacteroidales within time, not considering the type of treatment. **g** Differences (P=0.05) in Rikenellaceae relative abundance in patients considering the time and the type of treatment. **h** Differences in α-diversity index observed OTU (P<0.05) within time, not considering the type of treatment. **i** Differences in α-diversity index observed OTU (P<0.05) considering the healthy subjects (H) and patients at T2 subgrouped in spinal (S) and bulbar (B) onset.

Table 3 β–diversity comparisons between different groups of subjects.

Group 1	Group 2	*P*-value
T0 All diseased	T1 Group B	0.008
T0 All diseased	T2 Group B	0.024
T0 All diseased	T1 Group A	**0.001**
T0 All diseased	T2 Group A	**0.001**
T1 Group B	T2 Group B	**0.002**
T1 Group A	T2 Group A	0.005
T1 Group A	T2 Group B	**0.001**
T1 Group B	T2 Group A	**0.001**
T1 Group B	T1 Group A	0.047
T2 Group B	T2 Group A	**0.001**
H	T2 Group B	**0.001**
H	T2 Group A	**0.001**

The related *P*-values were calculated with Monte Carlo permutation test; bolded values indicate the adjusted significant *P*- value ($P < 0.003$), based on the Bonferroni correction for the comparisons performed.

Discussion

Pre-clinical studies demonstrated that gut microbiota composition is altered in murine ALS model [10,11] and an association between repeated antibiotics use and an increased risk of ALS has been reported as well [39]. To the best of our knowledge, only two studies have focused on the intestinal microbiota of ALS patients [12,13], collecting information on healthy and diseased subjects without reaching a definitive conclusion on gut dysbiosis in ALS and not considering possible variations of the microbiota with disease progression. The power of our study relies on: 1) the consistent number of subjects enrolled according to a case control method. Fifty phenotypically and genetically well characterized ALS patients and 50 matched healthy subjects were examined; 2) the consideration of possible modifications of the microbiota composition during the course of the disease performing multiple analysis during a six months period; 3) the supplementation for the first time of a probiotic formulation to ALS patients; 4) the microbiota analysis performed through an integrated molecular approach that includes PCR-DGGE, NGS and qPCR.

Baseline

The significantly lower DNA amount in ALS patients with respect to healthy controls is not due to a reduced bacterial count but can be ascribed, at least partially, to the count of total yeasts that was significantly higher in healthy controls. However, it cannot be excluded that epithelial cell DNA may influence the amount of total DNA, due to a higher epithelial cell turnover in healthy subjects with respect to ALS patients [40].

DGGE bacterial profiles showed a great variability both in healthy and diseased subjects. Their comparison does not show a clear shift in the intestinal microbiota composition in the diseased patients with respect to the healthy ones. This is also in agreement with the not significant alteration of the F/B ratio, considered by some authors as an indicator of dysbiosis [12]. However, the DICE-UPGMA analysis of DGGE profiles, mainly based on the presence/absence of bands, showed a well-defined cluster division, except for some samples, between diseased and healthy subjects. This analysis allowed us to conclude that differences in the predominant bacterial composition between healthy and diseased individuals do exist as also confirmed by the reduced α-diversity calculated with Chao1 index. The decrease in α-diversity was particularly evident comparing healthy and patients with lower ALSFRS-R score. Therefore, the patients with a greater disability showed a lower intra-individual diversity. Furthermore, the groups of healthy subjects and ALS patients resulted significantly different considering inter-individual diversity (β-diversity), as shown by the PCoA analysis. This difference was also evident in patients with higher ALSFRS-R score suggesting that inter-individual diversity is not related to the level of disability of the patients but to the intrinsic role of the disease in determining the bacterial diversity.

On the contrary, the DGGE yeast profiles did not show appreciable qualitative differences, although lower yeast levels were detected in patients than in controls. Interestingly, the yeast amount was higher in patients with a lower degree of disability, thus confirming the positive role of yeasts as gut beneficial commensals [41].

From the NGS analysis, Bacteroidetes and Firmicutes were the most abundant phyla, as expected [42], in both healthy and diseased subjects. Actinobacteria and Verrucomicrobia were higher in ALS patients. The higher abundance of Actinobacteria as well as of some families, such as Coriobacteriaceae, Lactobacillaceae, Bacteroidales S24-7 group belonging to Bacteroidaceae and some Clostridiales groups (Clostridiales vadin group BB60 and Clostridiales Family XI) were previously reported in major depressive disorder [43].

A very interesting result of our study is the demonstration that Cyanobacteria, at phylum level, were significantly more abundant in ALS patients compared to controls. This trend is also reflected at the family and genus level. These data are in agreement with the hypothesis that Cyanobacteria play a fundamental role in the pathogenesis of neurodegenerative diseases and particularly of ALS. The Cyanobacteria hypothesis emerged from studies carried out in Guam, which concluded that the non-protein aminoacid β-methylamino-L-alanine (BMAA), derived from a tropical plant, is likely responsible for a disease complex consisting of a sporadic form of ALS combined with PD and dementia (ALS/PDC) [44–49]. BMAA was found to originate from the symbiotic Cyanobacteria resident in specialized coralloid roots of *Cycas micronesica* and it biomagnified in the food chain through animals (flying foxes, pigs, deers) up to man. Murch et al. demonstrated that BMAA is concentrated in human brains of patients with ALS/PDC but not in control brains [49]. The mechanism of action of BMAA may be linked to the glutamate hypothesis of ALS, for which the exposure to excitatory aminoacids (glutamate and aspartate) stimulates glutamate receptors,

resulting in excessive intracellular calcium ion accumulation and consequently motor neuron death [50–54]. Furthermore, Cyanobacteria are responsible for the production of other toxins that may influence ALS origin and progression such as saxitoxin that can lead to paralysis of voluntary musculature [55,56], microcystins that are toxic for brain [57,58] and nodularin that cause cytoskeletal damage [59]. Our results support the role of neurotoxicity related to the larger amount of Cyanobacteria in the pathogenesis of ALS. On the contrary, the genera *Lactobacillus*, *Bifidobacterium* and *Odoribacter*, all known to metabolize glutamate, were more abundant in ALS patients. Higher amount of *Odoribacter* in ALS patients was also previously reported by Rowin et al. [13]. We can hypothesize that the increased relative abundance of these bacteria can represent an attempt by the microbiota to eliminate an excess of potential neurotoxic substances. The enhancement of this protective mechanism through probiotic supplementation can represent a new complementary therapeutic strategy.

Another important result of our study is the demonstration of the imbalance of some intestinal bacteria that play important roles in the immunomodulation of the central nervous system. The role of innate and adaptive immune response and inflammation in the pathogenesis of ALS is well known. Verrucomicrobia phylum, Verrucomicrobiaceae family and the Akkermansia genus, belonging to this family, are higher in ALS patients, as previously reported in multiple sclerosis patients [9]. *Akkermansia* has been correlated to proinflammatory pathways including up-regulation of genes involved in antigen-presentation, B- and T-cell receptor signalling, and activation of complement and coagulation cascades [60]. Hence a high amount of this microbial group may contribute to the inflammatory condition in ALS. Moreover, we found lower levels of Veillonellaceae in ALS patients than in controls as previously reported in multiple sclerosis [7]. Veillonellaceae are phylogenetically related to *Clostridium*, which induces regulatory T cells [61]. Therefore, the lower abundance of Veilloneaceae in patients can be linked to a compromised maintenance of immune homeostasis. The higher abundance of some genera belonging to Enterobacteriaceae, such as *Citrobacter* and *Escherichia-Shigella*, in our patients may contribute to intestinal inflammation [62,63]. The increase in faecal Enterobacteriaceae has been also reported in patients with major depressive disorders, accompanied by a low level of brain-derived neurotrophic factor (BDNF) in the blood [64,65].

Different trends were shown for some families belonging to Clostridiales. Within Clostridiaceae, the significantly lower amount of Clostridiaceae 1, including some ambiguous taxa belonging to this family, as well as *Clostridium* cluster I in ALS patients is in agreement with the results previously reported by Rowin et al. [13]. Differently, the Peptostreptococcaceae family (synonymous of Clostridium Family XI) were over represented in patients as also observed in the guts of colorectal cancer patients [66]. Shifts in the Clostriales profile have been also reported in children with neurodevelopmental dysfunctions, in particular autism spectrum disorders [67,68].
Members of Eubacteriaceae, in particular the *Eubacterium eligens* group, and of Ruminococcaceae, principally *Ruminococcus* and *Subdoligranulum* genera, which are involved in the degradation of plant cellulose and hemicellulose in the host [69], were found to be lower in our ALS patients than in controls Their depletion can be associated to a reduced production of short chain fatty acids (SCFAs) and, consequently, to a lower energy provision in the host. Interestingly, the depletion of *Ruminococcus* in ALS patients is in agreement with the results reported by Rowin et al. [13]. The *Eubacterium eligens* group, which we found more abundant in ALS patients, is able to produce equol, an estrogen derived from the metabolism of dietary daidzein [70]. Gut-derived equol is

known to have a neuroprotective activity [71]. Therefore, these bacteria may have a protective role against the progression of the disease, as shown by their higher relative abundance in patients with lower disability.
The higher levels of *Citrobacter* in patients may also be involved in the pathogenesis of ALS. This genus, in particular *Citrobacter rodentium*, has been demonstrated to exert a pathogenic mechanism similar to enteropathogenic and enterohaemorrhagic *E. coli* [72]. Moreover, *C. rodentium* infection in mice induces colitis and dysbiosis characterized by an overgrowth of *C. rodentium* and a reduction in the abundance and overall diversity of the resident microbiota [73]. Members of the *Pseudoflavonifractor* genus, more abundant in ALS patients, possess an anorectic function [74]. Therefore, this data can be linked to a losing weight trend and hypermetabolism associated to ALS [75].

Follow-up and Probiotic intervention
This session of the study was designed to monitor the microbiota composition in function of the clinical progression of the disease and to analyze the influence of a probiotic/placebo administration on the dysbiosis and the clinical parameters of the disease. The administered strains were selected for their capability of counteracting pathogens, such as some enterobacteria [17,18], *Candida* strains [15] and of inhibiting the release of pro-inflammatory cytokines [16].
Previous studies [76] have suggested that probiotic bacteria, besides restoring a possible microbial imbalance, can be considered as delivery vehicles for neuroactive compounds representing a possible therapeutic/preventive strategy in neurological diseases. Our results showed that no substantial alterations in the gut microbial composition could be associated to the administered probiotic treatment and no significant alterations in the dysbiosis indicator were observed. Only the Rikenellaceae family, one of the most represented microbial family in the gut and belonging to Bacteroidales, significantly increased with the 6 months probiotic treatment (Fig. 4g); as its relative abundance was significantly higher at T2 Group A with respect to T2 Group B, we can conclude that the duration of the probiotic treatment may influence the gut microbial composition. Moreover, since Rikenellaceae members are involved in propionate production [77], this probiotic administration may be able to affect SCFAs production. The Bacteroidaceae increase, although not significant, in both groups, but noticeably in Group A (Fig. 4d), may confirm that the longer probiotic treatment has a more marked influence on the microbiota composition with respect to the short intervention. An opposite trend was observed for Prevotellaceae, which also belong to Bacteroidales that decreased, although not significantly, in the group receiving the longer probiotic intervention. The decrease cannot probably be ascribed to the probiotic intervention but to a general progression of the disease, as already observed in PD [3].
The administered *Lactobacillus* strains may have acted against some Clostridales families (Christensellaceae, Clostridiales vadin BB60 group, Clostridiaceae and Lachnospiraceae) in agreement with a number of studies describing the effectiveness of *Lactobacillus* strains against *Clostridium* species, especially against cytotoxicity and adhesion to the gut epithelium of some *Clostridium* strains [78–80]. Through direct and indirect actions, co-administration of probiotics could prevent *Clostridium difficile* infection [81,82].
The decrease of Lachnospiraceae has also been observed in subjects with IBS [83]. Differently, Ruminoccoccaceae showed a fluctuating trend increasing after 3 months and then decreasing in

following 3 months regardless the treatment. As already reported, this is one of the main microbial groups showing differences between healthy and diseased subjects [12].
The Verrucomicrobiaceae decrease, although not significant, only in Group A, is in agreement with literature studies related to probiotic interventions against *Clostridium* infection in mice, which showed that Verrucomicrobiaceae are particularly sensitive to *Bifidobacterium* and *Lactobacillus* supplementation [84], and with studies on PD [3,85]. Therefore, the increase of this microbial group may be implicated in the pathogenesis of neurological diseases, as PD and ALS, and the probiotic formula experimented in this study may be effective in contrasting the variation of Verrucomicrobiaceae if administered for 6 continuous months.
Furthermore, 6 months-probiotic administration may have contrasted some microbial groups potentially harmful for the host, such as *E. coli*, *Clostridium* cluster I, enterobacteriaceae, as shown by qPCR analysis. The counts of total yeasts also decreased upon probiotic administration, thus showing that the probiotic formulation had no stimulating effects on this microbial group.
Some changes were observed considering only time as the variable and they can be associated with the progression of the disease. Bacteroidetes and related families were the microbial groups that changed the most with time. The significant increase after 3 months (T1) from baseline (T0) may represent a protective mechanism to counteract neurotoxicity during the progression of the disease. Bacteroidetes, representing a large part of the intestinal microbiota, are responsible for various important functions, such as the stimulation of T-cell mediated responses, butyrate production, as well as bile acid, toxic and/or mutagenic compounds metabolism [86].
The decrease of Cyanobacteria in both groups is not statistically significant. Since a positive correlation between Tenericutes and crude fiber digestibility has been shown in pigs [87], the significant decrease of this phylum after 3 months (T1) from baseline (T0) may be associated with a reduced fiber digestion and, therefore, a poorer nutritional status with the progression of the disease. Further analyses, including SCFAs investigation, are required to clarify the correlation between Tenericutes and diet in ALS progression.
In addition, the reduction of α-diversity, calculated as observed OTU, does not depend on the treatment but on the disease progression. The reduced α-diversity is also significant in ALS patients with spinal onset at T2 in comparison with healthy individuals. Both the progression and the kind of onset may affect the intra-individual biodiversity. The differences observed also in the β-diversity between the different groups may be attributed to the high inter-individual variability. It is important to highlight that the β-diversity changed upon the probiotic administration and since it is different in both treatments at T2 with respect to the baseline, it can be assumed that the duration of the probiotic treatment can have a remarkable influence on the microbiota biodiversity. In spite of these relevant changes, none of the interventions brought the gut microbiota biodiversity of ALS patients closer to that of healthy subjects and influence the progression of the disease.
This is the first study that clearly shows the modifications of gut microbiota composition in ALS patients by applying novel and very rigorous methodologies. This approach can be applied in larger clinical studies incorporating different genetic and phenotypical disease variants in order to characterize the microbiota changes as a novel biomarker of the disease. Moreover, our study represents a preliminary clinic probiotic application in ALS patients, a field of study that relies only on very few works mainly performed in animal models. Our study suggests that some effects can be obtained in contrasting potentially pathogenic microbial groups and, thus, poses the bases for a microbial strategy to ameliorate the health status of the gut. The knowledge derived from this study

can be applied in multicentric studies, creating a network involving a consistent number of ALS centers to test novel microbial strategies for attenuating the ALS phenotype and progression. However, our study has limitations. Firstly, twenty patients did not complete the study and one died, therefore, the restricted group size at T1 and T2 hampered the statistical power of sequencing data. Secondly, the intra-individual variability both in patients and controls, due to different lifestyles, has not been considered in the study. Thirdly, the patients were recruited according to strict inclusion criteria aimed at the clinical trial, hence an analysis of phenotypic subgroups is not feasible.

Conclusions

The results that we obtained, besides increasing knowledge on the gut microbiota of ALS patients, show that ALS is associated to a shift in the microbiota with respect to healthy individuals also in patients with low disability and full vital functions, suggesting the intrinsic role of the disease in the gut microbial alteration. We have demonstrated in this study that the gut microbiota composition changes during the course of the disease and can influence its progression. Some microbial groups such as Cyanobacteria may facilitate the pathogenesis of the disease, influencing the inflammatory cytokines and bacterial metabolites production, while others may provide neuroprotection against the progression of the disease.

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Additional file 1

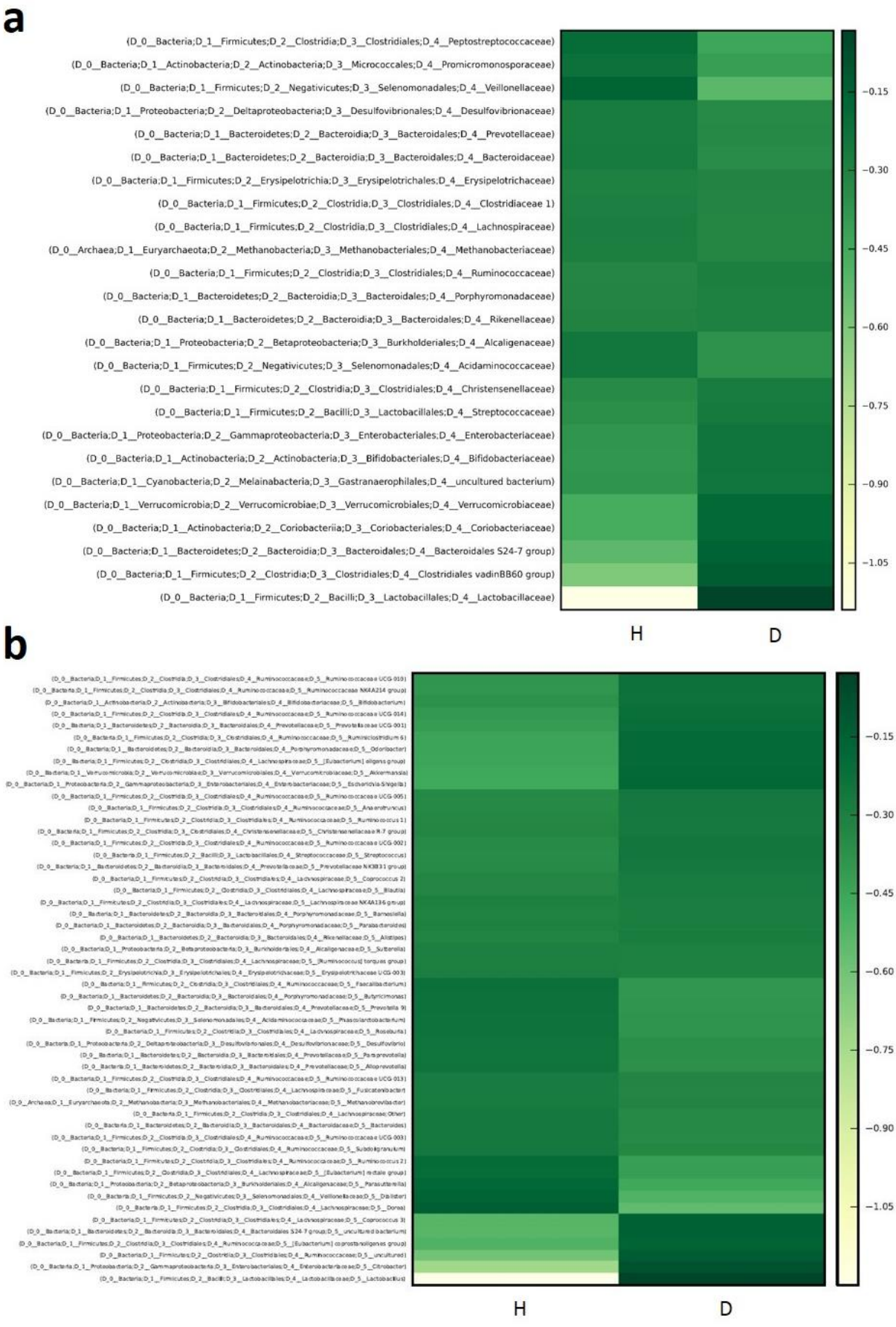


Figure S1. Gut bacterial families and genera characteristics at baseline. a**)** Relative abundance of microbial groups at family level in the healthy group (H) and diseased group (D); families with a relative abundance less than 0.002 are omitted for the sake of clarity. b) Relative abundance of microbial groups at genus level in the healthy group (H) and diseased group (D); genera with a relative abundance less than 0.003 are omitted for the sake of clarity.

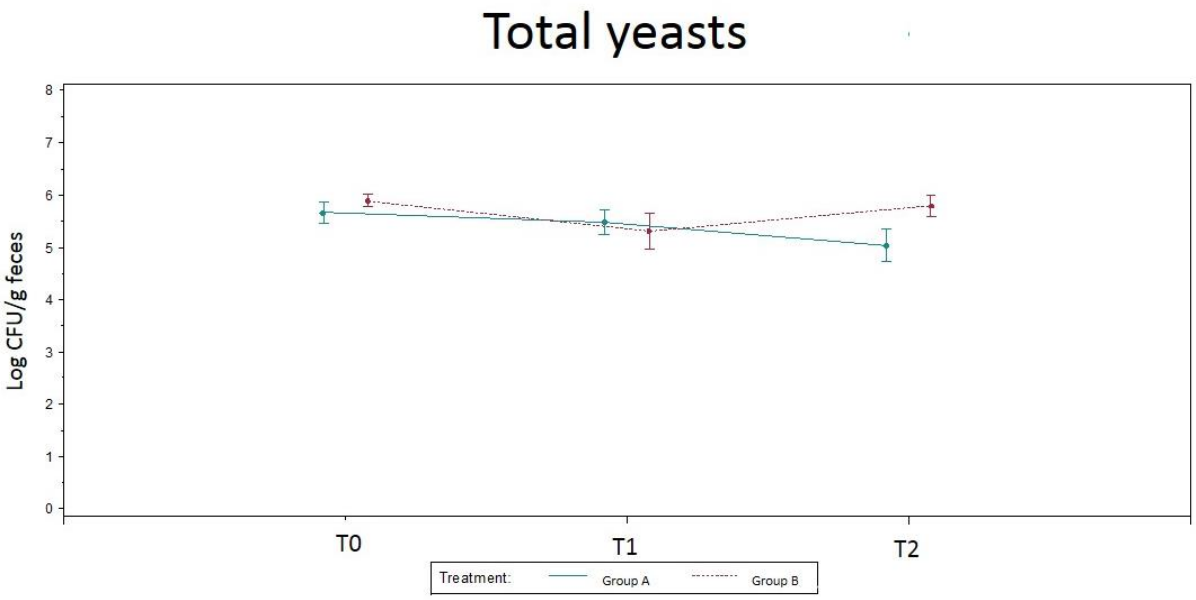


Figure S2. Fecal yeast qPCR counts during the intervention. The graphic was conceived as mean plot reporting also the standard errors.

Table S1. DNA concentration and mean counts of the analyzed microbial groups by qPCR in stool samples of ALS patients during the intervention.

	Time	**Group A**		**Group B**		***P*-value**
		n.	Mean (SD)	n.	Mean (SD)	
DNA	T0	24	153.58 (120.16)	26	157.27 (119.65)	0.91
	T1	16	132.44 (70.37)	13	120.61 (56.88)	0.63
	T2	13	90.99 (54.9)	12	116.5 (52.42)	0.25
Total bacteria	T0	24	10.33 (0.86)	26	10.38 (0.87)	0.91
	T1	16	10.46 (0.71)	13	10.15 (1.13)	0.38
	T2	13	10.45 (0.48)	12	10.67 (0.64)	0.34
***Lactobacillus* spp.**	T0	24	5.4 (1.54)	26	5.48 (0.96)	0.54
	T1	16	5.61 (1.34)	13	5.21 (1.27)	0.36
	T2	13	5.33 (1.28)	12	5.47 (0.78)	0.94
***Bifidobacterium* spp.**	T0	24	7.07 (1.82)	26	7.52 (1.43)	0.42
	T1	16	7.73 (0.95)	13	7.47 (1.85)	0.98
	T2	13	6.78 (1.91)	12	7.79 (0.89)	0.24
E. coli	T0	24	6.64 (1.17)	26	6.55 (1.11)	0.78
	T1	16	6.04 (1.9)	13	6.23 (1.65)	0.77
	T2	13	5.66 (1.95)	12	6.93 (0.92)	**0.05**
***Clostridium* cluster I**	T0	24	5.53 (1.3)	26	5.88 (1.76)	0.27
	T1	16	5.48 (1.41)	13	5.72 (1.36)	0.63
	T2	13	4.97 (1.16)	12	5.61 (0.81)	0.12
Enterobacteriaceae	T0	24	8.55 (0.78)	26	8.48 (0.82)	0.76
	T1	16	8.13 (1.83)	13	8.08 (1.97)	0.95
	T2	13	7.71 (2.07)	12	8.75 (0.56)	0.11
Total Yeasts	T0	24	5.66 (1)	26	5.89 (0.6)	0.32
	T1	16	5.48 (0.97)	13	5.32 (1.24)	0.84
	T2	13	5.04 (1.09)	12	5.8 (0.7)	**0.03**

DNA concentrations are expressed as ng DNA/200 mg of faeces and the mean counts as Log CFU/g of feces, the related P- value is reported. Bolded values indicate $P < 0.05$.

Table S2. Relative abundance of the main families for each group of subjects.

Families	Healthy	T0 Group A	T1 Group A	T2 Group A	T0 Group B	T1 Group B	T2 Group B
Methanobacteriaceae	0.637	0.394	0.184	0.804	0.782	1.572	0.517
Actinomycetaceae	0.009	0.009	0.006	0.015	0.011	0.010	0.009
Bifidobacteriaceae	2.073	1.191	2.191	1.257	4.486	4.147	5.817
Promicromonosporaceae	0.287	0.169	0.152	0.452	0.208	0.2583	0.175
Coriobacteriaceae	0.223	0.378	0.439	0.989	0.476	0.498	0.722
Bacteroidaceae	24.109	24.114	29.404	32.328	16.085	25.195	25.240
Bacteroidales S24-7 group	1.038	1.328	0.590	0.246	3.451	1.742	2.498
Porphyromonadaceae	5.318	5.262	8.682	8.685	5.820	7.763	4.580
Prevotellaceae	8.086	5.896	3.682	1.910	8.310	9.881	6.514
Rikenellaceae	5.965	4.851	8.732	12.249	7.22	7.144	4.823
Bacteroidales; uncultured bacterium	0.080	0.094	0.074	0.048	0.164	0.388	0.177
Bacteroidales;Other	0.007	0.007	0.021	0.007	0.009	0.010	0.004
Gastranaerophilales;uncultured bacterium	0.173	0.351	0.019	0.102	0.114	0.088	0.013
Lactobacillaceae	0.048	0.142	0.068	0.034	1.091	0.036	0.045
Streptococcaceae	0.362	0.609	0.266	0.212	0.286	0.213	0.217
Christensenellaceae	2.462	2.816	2.016	1.225	2.908	3.118	2.393
Clostridiaceae 1	0.248	0.163	0.108	0.025	0.307	0.200	0.070
Clostridiales vadinBB60 group	0.104	0.265	0.198	0.061	0.365	0.130	0.270
Defluviitaleaceae	0.015	0.012	0.024	0.012	0.032	0.019	0.011
Clostridiales;Family XIII	0.146	0.225	0.533	0.212	0.156	0.195	0.104
Lachnospiraceae	11.484	10.518	9.020	7.588	10.443	7.980	9.167
Peptococcaceae	0.114	0.111	0.105	0.105	0.139	0.256	0.108
Peptostreptococcaceae	0.443	0.250	0.109	0.104	0.266	0.153	0.092
Ruminococcaceae	20.908	23.617	18.248	22.755	21.137	17.813	21.021
Clostridiales;Other	0.018	0.012	0.015	0.008	0.019	0.019	0.012
Thermoanaerobacteraceae	0.050	0.039	0.065	0.040	0.031	0.017	0.022
Erysipelotrichaceae	0.887	1.102	0.310	0.558	0.637	0.515	0.402
Acidaminococcaceae	2.265	1.643	1.666	1.230	1.836	2.237	2.085
Veillonellaceae	4.250	1.347	2.837	1.448	2.390	0.886	1.206
Fusobacteriaceae	0.002	0.007	0.011	0.016	0.005	0.007	0.029
Victivallaceae	0.005	0.015	0.002	0.011	0.014	0.007	0.025
Rhodospirillaceae	0.129	0.304	0.474	0.506	0.056	0.227	0.202
Oxalobacteraceae	0.020	0.044	0.016	0.023	0.021	0.047	0.058
Desulfovibrionaceae	0.680	0.817	0.543	0.525	0.368	0.445	0.526
Enterobacteriaceae	1.717	1.656	2.463	0.752	3.025	1.243	3.190
Pasteurellaceae	0.345	0.029	0.026	0.008	0.036	0.027	0.032
Synergistaceae	0.128	0.327	0.304	0.159	0.071	0.118	0.398
Mollicutes RF9; uncultured bacterium	0.016	0.035	0.002	0.022	0.075	0.004	0.029
Mollicutes; NB1-n;Ambiguous taxa	0.012	0.012	0.002	0.004	0.049	0.008	0.005
Opitutae vadinHA64; uncultured bacterium	0.022	0.042	0.006	0.001	0.051	0.039	0.055
Verrucomicrobiaceae	3.557	7.937	4.409	1.067	5.562	3.643	5.994

Data are expressed as percentage of relative abundance; families with a relative abundance less than 0.001% are omitted for the sake of clarity.

Paper 7

Study of the intestinal bacteria and bacteriophage composition in preterm neonates

This work has been conducted in collaboration with the Neonatal Intensive Care Unit (NICU) of Azienda Ospedaliero-Universitaria of Modena (Italy) and the Department of Food Science of the University of Copenhagen (Denmark)

This paper has not been completed yet and it needs finalization prior to publication

Abstract

Prematurity in infancy is a clinical condition that exposes newborns to the risk of infections that can result in the development of necrotizing enterocolitis and late onset sepsis. Therefore, preterm infants often require antibiotic therapies at birth and during the hospitalization that can affect the correct establishment of the gut microbiota. Recent results highlighted the potential of bacteriophages in driving the development of gut microbiota, suggesting a transkingdom interplay between bacteria and viruses.

The aim of this study was to investigate the gut bacteria and bacteriophage composition in preterm neonates, who have been treated with perinatal antibiotic treatments of different duration (≤72h or >72h). Stool samples were longitudinally collected within the first 48 hours after birth, and at 15, 30 and 90 days of life. Total bacterial genome and phageome were extracted. V3 region of 16S rDNA and the total phageome were sequenced using the MiSeq Illumina facilities. Absolute quantification of selected microbial groups by qPCR was also performed.

Changes in bacterial composition, especially for Enterobacteriaceae, *Bifidobacterium*, *Streptococcus* and *Veillonella*, and in bacteriophages composition, especially for *Pahexavirus*, *Lambdavirus* and *Lubbockvirus* were detected over time. The biodiversity of the bacterial and bacteriophages community increased simultaneously, but at 90 days, the alpha diversity diverged, probably for the establishment of microbial groups that did not support the development of bacteriophages. Some typical disease conditions of prematurity, as respiratory distress, sepsis and cardiac malformation affected the microbiota composition, in particular *B. fragilis*, *Bifidobacterium* and *Peduovirus*.

The biodiversity of bacterial and bacteriophages communities were also differently influenced by the short (≤72h) or prolonged (>72h) perinatal antibiotic therapy, although not with a statistical significance. In addition, the longer duration of the perinatal antibiotic treatment reduced, although without statistical significance, the diversification of two of the more representative members of the infant gut, *Bifidobacterium* spp. and *Bacteroides fragilis* group, and decreased bacteriophages integrase counts, favouring potentially lytic phages.

These findings on gut microbiome and phageome and their interplay may help to design a targeted microbial approach for the correct development of gut microbiota in premature infants and prevent disease onset later in life.

Keywords: preterm infants, gut microbiota, bacteriophages, microbial biodiversity, antibiotics

Introduction

The gut microbiota, impacting on several biological processes, such as host metabolism, immune defence and protection against pathogens, establishes a symbiotic relationship with the host contributing to the normal human physiology (Wopereis et al., 2014; Morais et al., 2017; Clarke et al., 2014). The neonatal period is a crucial stage for the correct gastrointestinal colonization that has an impact on the individual's health status also later in life (Sekirov et al 2010).

Infants born prematurely have a gut microbiota with reduced diversity and lower levels of *Bifidobacterium* and *Bacteroides* compared to full-term infants (Barrett et al., Korpela et al., 2018). The typical gut colonization sequence in healthy infants, consisting of the predominance of facultative anaerobes firstly and strict anaerobes secondly, seems to be compromised in prematures. In this regard, Gibson et al. (2016) demonstrated that at 1 and 2 months from delivery the gut microbiota of preterm infants hosts a higher abundance of Enterobacteriaceae and Staphylococcaceae, which are facultative anaerobes, and a lower abundance of Clostridiaceae and Bidobacteriaceae, which are strict anaerobes, together with Streptococcaceae and Lachnospiraceae with respect to that of full-term infants. The same authors evidenced higher abundance of multidrug-resistant members of the genera *Escherichia, Klebsiella* and *Enterobacter*, commonly associated with nosocomial infection, in the preterm infant gut (Gibson et al., 2016).

Moreover, preterm infants, requiring parental feeding and respiratory support for their underdeveloped gastrointestinal tract and respiratory system and being vulnerable to infections, often need antibiotic therapies. It is known that perinatal antibiotics impact on the normal development of the gut microbiota (Fouhy et al., 2012). Gibson et al. (2016) documented that preterm infants exposed to antibiotics showed a significantly reduced species richness. An altered and underdeveloped intestinal microbiota can affect the host's immunity status and increase the risks of developing necrotizing enterocolitis (NEC) and late onset sepsis (LOS) (Underwood and Sohn, 2017).

Bacteriophages, other inhabitants of the human and animal intestinal tract, living at bacteria expense and being vehicles of genetic transfer, can have an important role in shaping the biodiversity and therefore the functionality of the gut ecosystem. Our knowledge of their role in shaping the microbial community of infants is, however, limited, and only recently we gained a better understanding of this microbial community. Breitbart et al. (2008) investigated the bacteriophage community in the infant intestine using metagenomic sequencing: 72% of the viral community detected resulted to be siphoviruses and prophages and over 25% resulted to be phages that infect lactic acid bacteria. The virome is highly dynamic during infancy: in the first months of life, the majority of bacteriophages belongs to Caudovirales; subsequently, the bacteriophage virome decreases in richness and shifts towards a Microviridae-dominated community over the first 2 years of life (Reyes et al., 2015; Lim et al., 2015). This shifting trend between the first and second week of age was also confirmed by microarray experiments (Breitbart et. al 2008).

Bacteriophages can alter the bacterial structure through a predator–prey relationship, and particularly frequent in the infant gut is the bifidobateria-bifidoprophages predator-prey interplay (Norman et al., 2015; Rodriguez-Valera et al., 2009; Lugli et al., 2015). Moreover, early-life bacterial colonization is correlated with a contraction of bacteriophages community (Lim et al., 2015).

Some authors support an intestinal microbial model in which changes in the phageome may contribute to intestinal inflammation and bacterial dysbiosis, speculating that bacteriophages may

play a role in diseases not only related to the intestinal tract (Norman et al. 2015). Gut bacteria and their genome-integrated prophages are able to coexist in a passive state under normal physiological conditions, but the induction of prophages in response to various environmental stresses, such as antibiotics, leads to an increased number of lytic phages followed by the lysis of symbiotic bacteria resulting in disturbed balance of the gut microbiota (Allen et al., 2011).
Only two studies reported some preliminary information about the bacteriophages population in gut microbiota of prematures. LaTuga et al. (2011) performed a metagenome analysis on two newborn faecal samples revealing the presence of both single-strand, including bacteriophages S13, phiX174, and alpha 3, and double strand DNA viruses, including *Staphylococcus* Phage K, a Caudovirales species with a *Staphylococcus* host; a rare human adenovirus C sequence was also identified. These results are in accordance with Sharon et al. (2013), who, reconstructing genome sequences of intestinal microorganisms of a premature infant, observed a rapid change in the gut phage population as shown by the marked shifts in the relative frequency of phages associated with some *Staphylococcus epidermidis* strains during gut colonization.
Bacteriophage investigation can provide potential informative signatures of the whole gut microbiota community and gut microbial responses to different disturbances (Reyes et al., 2010), such as antibiotics, and their role in preterm infants should be more extensively investigated.
The aim of this study was to investigate the whole bacterial composition of the intestinal tract of extremely preterm infants as well as the related bacteriophage community. Particularly, the effects deriving from perinatal antibiotic therapies of different duration (≤72h or >72h) on these microbial communities were studied.

Materials and methods

Study Design and Participants

We conducted a prospective observational cohort study involving 23 preterm infants admitted to the Neonatal Intensive Care Unit (NICU) of Azienda Ospedaliero-Universitaria of Modena (Italy). Informed consent was obtained prior to enrolment from parents of each case included in the study. In order to assess faecal microbiota profiles, 23 preterm infants were longitudinally sampled within the first 48 hours (T0) after birth, and at 15 (T1), 30 (T2) and 90 (T3) days of life. Infants received either a short (≤ 72 hours) or a prolonged course (> 72 hours) of postpartum antibiotics. Exclusion criteria were: major malformations, gestational age over 28 weeks or the administration of probiotics.
From August 2017 to September 2018 stool swabs were collected from the diapers of NICU patients and subsequently stored at -80°C.

Analysis of intestinal bacterial composition

Total DNA extraction
Meconium and faecal samples were subject to DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) with a slight modification of the standard protocol: a supplementary incubation at 95◦C for 5 min of the stool sample with the lysis buffer was added to enhance the bacterial cell rupture. The purity of DNA was determined by measuring the ratio of the absorbance at 260 and 280nm (Infinite R 200 PRO Nano Quant, Tecan, Mannedorf, Switzerland) and the

concentration was evaluated by Qubit R 3.0 Fluorometer (Invitrogen, Life Technologies, CA, USA).

Library preparation for V3 16S rDNA sequencing

DNA samples were subjected to 16S ribosomal RNA gene MiSeq-based high throughput sequencing (Illumina, San Diego, CA, USA). The V3 region (~190bp) of the 16S rRNA gene was amplified using primers compatible with Nextera Index Kit (Illumina): NXt_388_F: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCAGCAG-3′ and NXt_518_R: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG-3′ (Integrated DNA Technologies; Leuven, Belgium). PCR reactions contained 0.25 µl of PCRBIO polymerase, 5 µl of PCRBIO buffer (PCR Biosystems Ltd., London, UK), 0.5µl of each primer (10 µM), 5µl of genomic DNA (~1– 10ng/µl), and nuclease-free water for a total volume of 25µl. Cycling conditions applied were: 95°C for 2min; 33 cycles of 95°C for 15s, 55°C for 15s, and 72°C for 20s; followed by final step at 72°C for 4min. Agarose gel electrophoresis was performed to ensure that a 250-bp PCR product was obtained for each sample, except negative control. To incorporate primers with adapters and indexes, PCR reactions contained 0.25 µl of PCRBIO polymerase, 5 µl of PCRBIO buffer, 2µl corresponding P5 and P7 primer (Nextera Index Kit), 2µl PCR product, and nuclease-free water for a total volume of 25µl. Cycling conditions were: 95°C for 1 min; 13 cycles of 95°C for 15s, 55°C for 20s, and 72°C for 15s; and 72°C for 5min. The amplified fragments with adapters and tags were purified using AMPure XP beads (Beckman Coulter Genomic, CA, USA). Prior to library pooling, clean constructs were quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and mixed in approximately equal concentrations to ensure even representation of reads per sample. Each sample was represented with approximately 30ng DNA. Subsequently, 250-bp pair-ended MiSeq (Illumina, CA, USA) sequencing was performed according to the instructions of the manufacturer.

Absolute quantification of selected microbial groups using quantitative PCR (qPCR)

Quantification of selected microbial groups usually monitored in studies related to infants (Aloisio et al., 2014; Aloisio et al., 2018), i.e., *Bidobacterium* spp., *Bacteroides fragilis* group (comprising the most abundant species in human *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. vulgatus*), *Clostridium* cluster I and Enterobacteriaceae, was performed with qPCR on DNA extracted from stool samples. The assays were carried out with a 20 µL PCR amplification mixture containing 10 µL of Fast SYBR R Green Master Mix (Applied Biosystems, Foster city, CA, USA) optimized concentrations of primers (**Tables 1, 2**), molecular grade H_2O and 2 µL DNA obtained from faecal samples at a concentration of 2.5 ng/µL. The primer concentrations were optimized through primer optimization matrices in a 48-well plate and estimating the best Ct/1Rn ratio. The different primers were also checked for their specificity utilizing the database similarity search program nucleotide-nucleotide BLAST (Altschul et al. 1990). Moreover, to evaluate the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification. The data obtained from the amplification were then converted to obtain the number of bacterial cells (Log CFU/g faeces) in accordance with the rRNA copy number available at the rRNA copy number database (Lee et al. 2009). For total bacteria, the average of the 16S rRNA genes calculated on 10996 records for bacteria according to rrDB was used as the rRNA copy number (rrndb.umms.med.umich.edu). Standard curves were constructed using 16S rRNA PCR

products of type strains of each target microorganism; the standard microorganisms used were *B. breve* ReO2, *B. fragilis* DSM 2151, *Clostridium sporogenes* ATCC 319 and *E. coli* ATCC 8739. PCR products were purified with a commercial DNA purification system (NucleoSpin R Extract II kit, MACHEREY-NAGEL GmbH & Co. KG, Germany) and the concentration measured spectrophotometrically at 260 nm. Serial dilutions were performed and 10^2,10^3,10^4,10^5,10^6,10^7 copies of the gene per reaction were used for calibration. Sample reactions were conducted in triplicate, with a negative control per each reaction.

Table 1. Primer sequences and qPCR conditions used in the different assays.

Microorganism target	Primer	Sequence (5'-3')	Amplicon length(bp)	References
Bifidobacterium spp.	Bif-F Bif-R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	243	Rinttila et al., 2004
B. fraglis group	Bfra-F Bfra-R	CGGAGGATCCGAGCGTTA CCGCAAACTTTCACAACTGACTTA	92	Penders et al., 2006
Clostridium cluster I	CI-F CI-R	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	232	Song et al., 2004
Enterobacteria	Ent-F Ent-R	ATGGCTGTCGTCAGCTCGT CCTACTTCTTTTGCAACCCACTC	385	Leser et al., 2002
Total bacteria	Eub338F Eub518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	200	Guo et al., 2008

Table 2. qPCR amplification protocols and primer concentrations

Taget Bacteria	Initial denaturation	Denaturation	Annealing t (°C)	N. cycles	Fw	Rev
Bifidobacterium spp.	95°C – 20sec	95°C - 3 sec	60°C - 35sec	40	200 nM	300 nM
B. fragilis group	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	200 nM	200 nM
Clostridium cluster I	95°C – 20sec	95°C - 3 sec	60°C - 30 sec	40	200 nM	200 nM
Enterobacteria	95°C – 20sec	95°C - 3 sec	60°C - 35sec	40	200 nM	300 nM
Total bacteria	95°C – 20sec	95°C - 3 sec	60°C - 35sec	40	200 nM	300 nM

Data elaboration

Several bioinformatics pipelines were used to analyse the amount of sequencing data. Sequences read processing was performed using QIIME suite of tools (Caporaso et al. 2010), including quality filtering based on the removal of mismatched barcodes and sequences under length thresholds; USEARCH version 7 (Edgar, 2010) was applied for chimera detection and clustering into operational taxonomic units (OTU); OTU sequences were aligned using Pynast (Caporaso et al. 2010) and taxonomic assignment was determined using the SILVA SSu Ref database release 111. The non-parametric Kruskal–Wallis was used to calculate group significance for alpha diversity and and ANOSIM and PCoA were used for beta diversity.

qPCR data were subjected to Shapiro and Levene tests in order to verify the normal distribution and homogeneity of variances. Statistical significance was evaluated with two-way ANOVA. Non-

normal and non-homoscedastic datasets were analysed with Mann–Whitney–Wilcoxon, Kruskall-Wallis and Dunn's Multiple Comparison Tests

Analysis of intestinal bacteriophages

Viral particles genome extraction

The procedure firstly consisted of isolating, purifying and concentrating viral particles from faecal samples by sequential centrifugation and filtration processes according to the protocol optimized by Castro-Mejia et al. (2015). Viral genome was extracted using the QIAamp® Viral RNA kit (Qiagen, West Sussex, UK), including an additional treatment with nuclease 1% to remove possible external DNA residuals. The phageome was then subjected to multiple displacement amplification (MDA), using the GenomiPhi V3 kit (GE Healthcare, Illinois, USA). The products were cleaned with Genomic DNA Clean & Concentrator™ -10 kit (Zymo Research, Irvine, California, USA) following the manufacturer's instructions. The DNA concentration was estimated by Qubit® 3.0 Fluorometer.

Library preparation for metagenome sequencing

The phageome was prepared for sequencing using the Nextera XT DNA Library preparation kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The library was cleaned using the AMPure beads XP purification system, quantified using the Qubit® 3.0 Fluorometer and pooled in an equimolar mode. DNA samples were subjected to a metagenome analysis by MiSeq-based high throughput sequencing (Illumina, San Diego, CA, USA).

Data elaboration

The composition of extracted metaviromes was determined using the MetaVir web server (Roux et al., 2014). Composition and relative abundance generated by Metavir were converted into OTU tables and subsequent analyses were performed using QIIME suite of tools (Caporaso et al. 2010). The differences in the relative abundance of phage-taxa were assigned using one-way ANOVA; cluster richness analysis will be carried out with UCLUST pipeline (Edgar et al. 2010). The non-parametric Kruskal–Wallis was used to calculate group significance for alpha diversity and and ANOSIM and PCoA were used for beta diversity.

Results and discussion

Intestinal bacterial composition

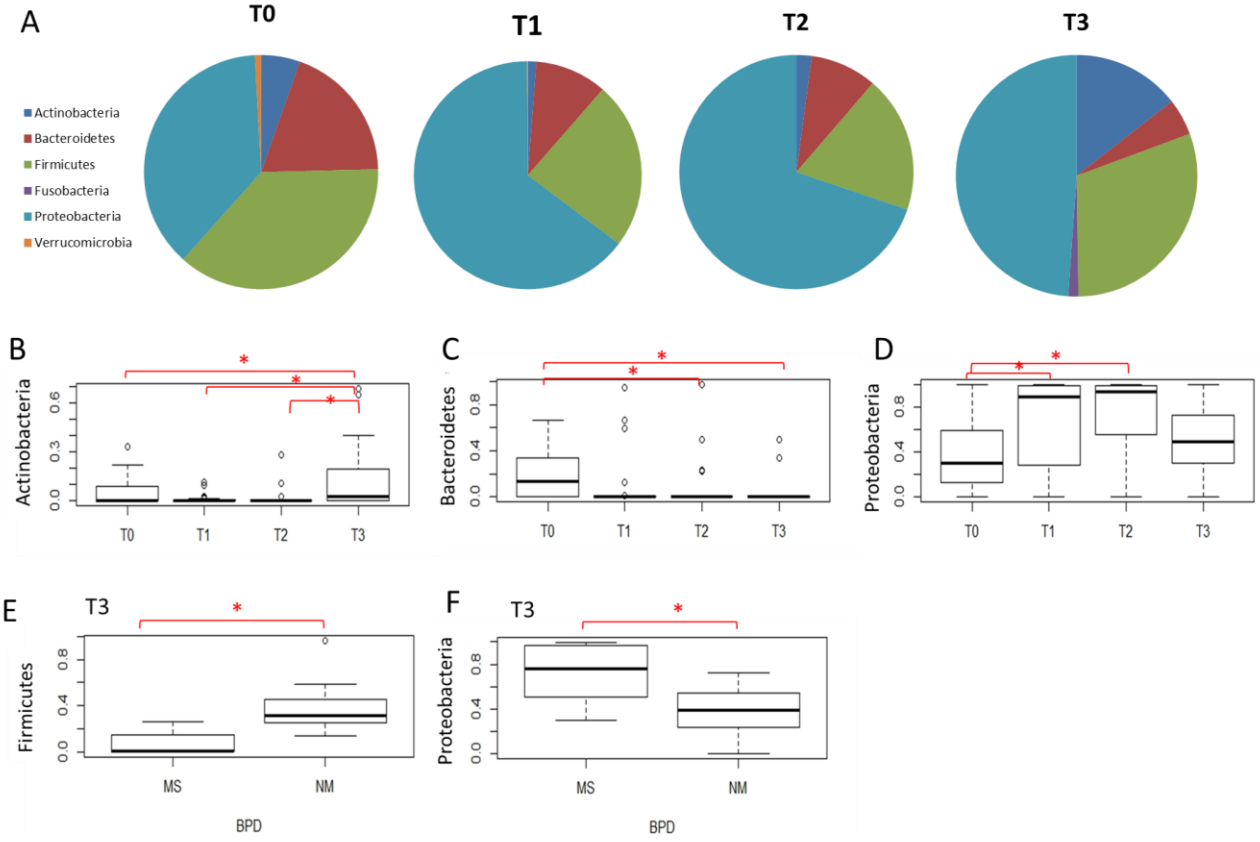
Sequencing data

A total of 84 DNA samples were subjected to the sequencing process and an average of 12 040 sequences per sample was generated. As shown in **Fig. 1A**, at T0, T1 and T2 Firmicutes, Proteobacteria and Bacteroidetes resulted the most abundant phyla. Compared to T0, Proteobacteria significantly increased at T1 and T2 (**Fig. 1D**) and Bacteroidetes decreased, particularly at T2 (**Fig. 1C**); as evidenced in **Fig. 1A,** the prevalence of Proteobacteria reduced at T3 in favour of the increase of Firmicutes, Actinobacteia (**Fig. 1B**), which were significantly higher at T3 with respect

to the other timepoints (**Fig. 1B**), and Fusobacteria, for which the increase at T3 with respect to T0 and T1 resulted statistically significant ($p<0.05$, data not shown).

Infants were grouped for the degree of bronchopulmonary dysplasia (BPD): MS group showed a moderate/severe condition and NM group did not manifest the disease or had a mild form. At T3, significantly lower levels of Firmicutes members, which include a number of beneficial bacteria, and significantly higher levels of Proteobacteria, which include potentially harmful bacteria, were found in MS group with respect to NM (**Fig. 1E** and **1F**). This trend may evidence an alteration of gut microbiota composition that shifts in favour of potentially harmful bacteria when the BPD is moderate/severe and of beneficial ones when the manifestation is mild or absent.

Fig. 1. Gut bacterial relative abundance at phylum level in faecal samples collected at different timepoints (A). Actinobacteria relative abundance at different timepoints (B). Bacteroidetes relative abundance at different timepoints (C). Proteobacteria relative abundance at different timepoints (D). Firmicutes relative abundance at T3 in moderate/severe (MS) and absent/mild (NM) BPD (E). Proteobeacteria relative abundance at T3 in moderate/severe (MS) and absent/mild (NM) BPD (D). "*" indicates $p<0.05$



From **Fig. 2** it is possible to appreciate a high inter-individual variability in microbial composition that is probably linked to the influence of environmental factors in the very first days of life, such as antibiotic therapies, type of feeding, respiratory support. The general high prevalence of Proteobacteria reflected the high abundance of Enterobacteriaceae detected at family level (**Fig. 2)**, which notably fluctuated over time, significantly increasing at T2 and decreasing at T3 (**Fig. 3A**). The trend of Actinobacteria was confirmed by Bifidobacteriaceae and *Bifidobacterium* one, which were reduced at T1 and T2 and increased at T3 (**Fig. 2, Fig. 3B**). These bacterial groups usually prevail in the gut microbiota at this period of infancy (Benno et al., 1984; Magne et al., 2006). The first bacteria encountered in the majority of healthy infants are facultative anaerobes (Rotimi and

Duerden, 1981; Mackie et al., 1999); in our case, as shown in **Fig. 2**, the presence of some strictly anaerobes (i.e. Bifidobacteriaceae, Clostridiaceae, Akkermansiaceae) was also evident in some samples at T0. It is possible that the condition of prematurity, the frequent feeding and the antibiotic exposure could create an environment favourable to the growth of these bacterial groups. However, they tended to decrease at T1 and T2 and then to prevail at T3.

Other significant changes were observed for other genera (**Fig. 3**), including some pathogens responsible of sepsis' onset: *Enterobacter* increased at T3 with respect to T0 (**Fig. 3C**); *Enterococcus* and *Staphylococcus* were more abundant within the first 15 days of life (**Fig. 3D** and **3E**); *Streptococcus* and *Veillonella* were higher at T3 with respect to the other timepoints (**Fig. 3F** and **3G**).

Moreover, the trend observed on BPD manifestation at phylum level was confirmed at genus level. *Escherichia*, belonging to Proteobacteria and resulting significantly higher in MS infants with respect to NM ones (**Fig. 3H**), may be considered an indicator for the severity of this disease. On the contrary, *Clostridium*, belonging to Firmicutes and resulting less abundant in MS infants (**Fig. 3I**), may be considered a protective factor for this condition.

Fig. 2. Gut bacterial families relative abundance in preterm infants during the study.

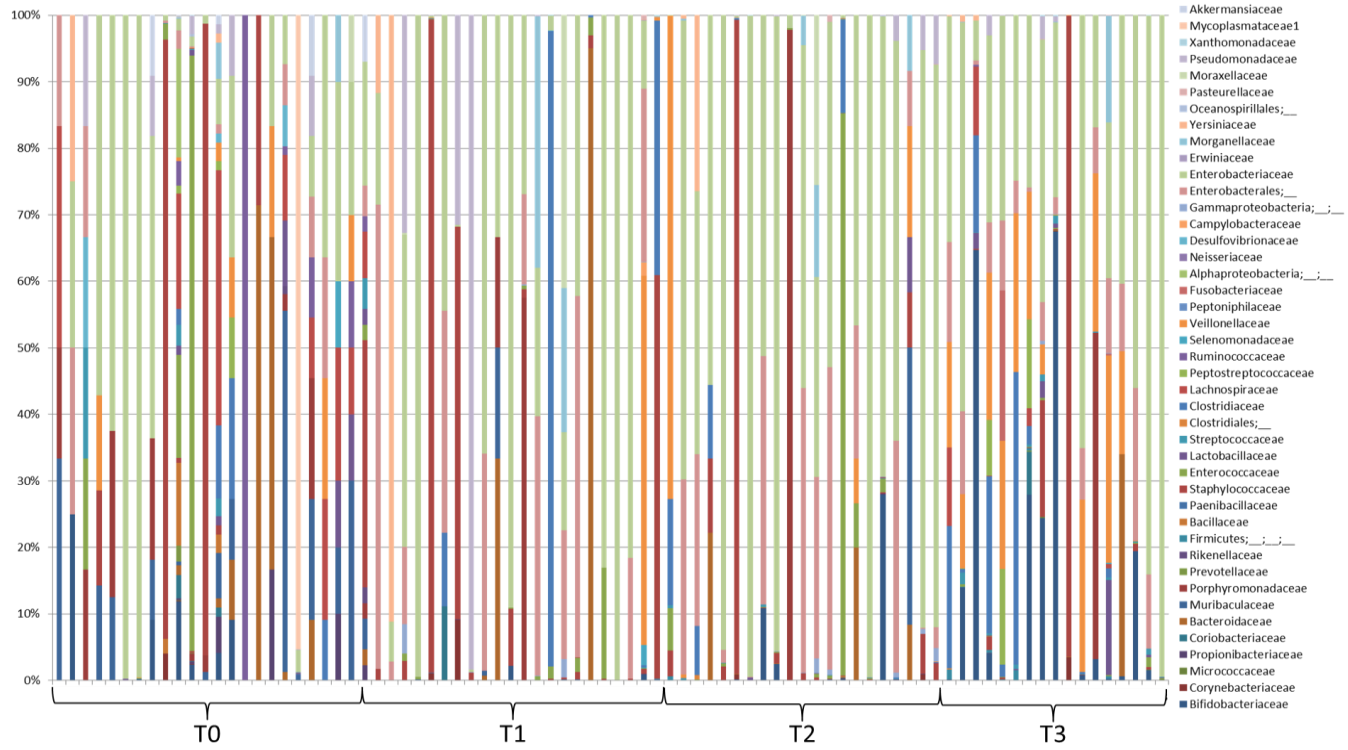
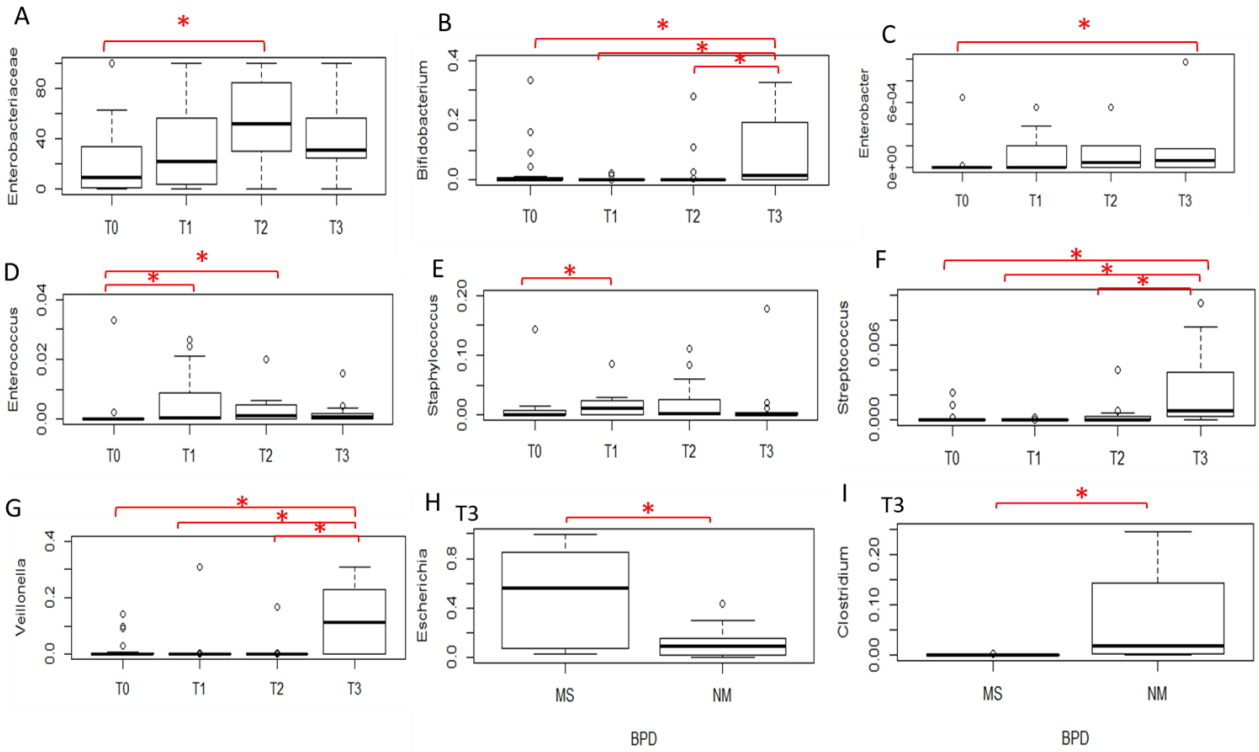
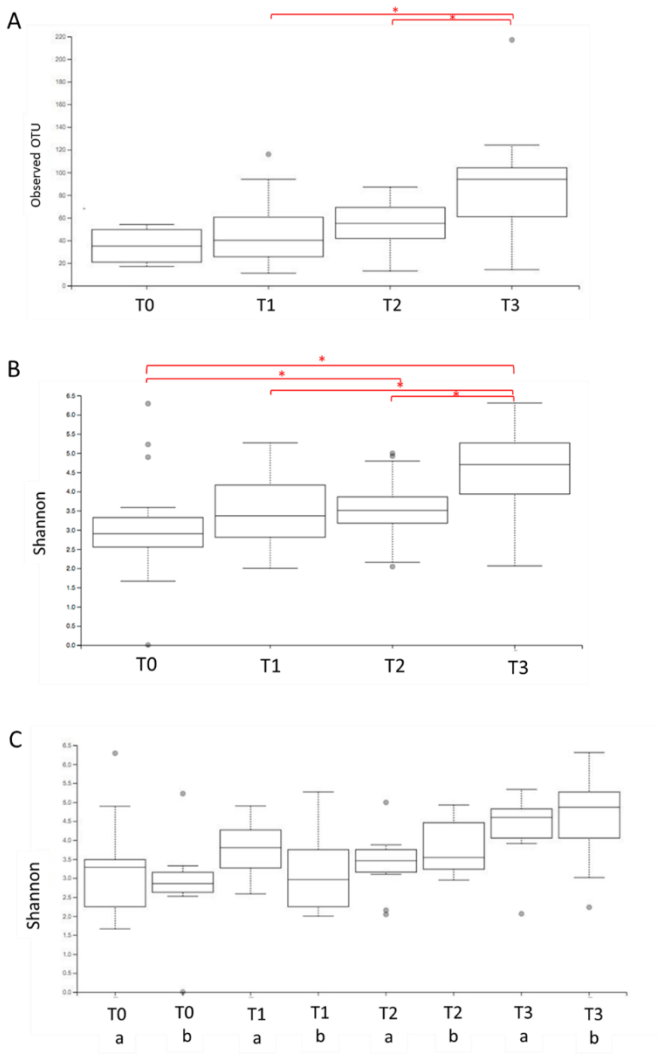


Fig. 3. Enterobacteriaceae relative abundance at different timepoints (A). *Bifidobacterium* relative abundance at different timepoints (B). *Enterobacter* relative abundance at different timepoints (C). *Enterococcus* relative abundance at different timepoints (D). *Staphylococcus* relative abundance at different timepoints (E). *Streptococcus* relative abundance at different timepoints (F). *Veillonella* relative abundance at different timepoints (G). *Escherichia* relative abundance at T3 in moderate/severe (MS) and absent/mild (NM) BPD (H). *Clostridium* relative abundance at T3 in moderate/severe (MS) and absent/mild (NM) BPD (I). "*" indicates $p<0.05$



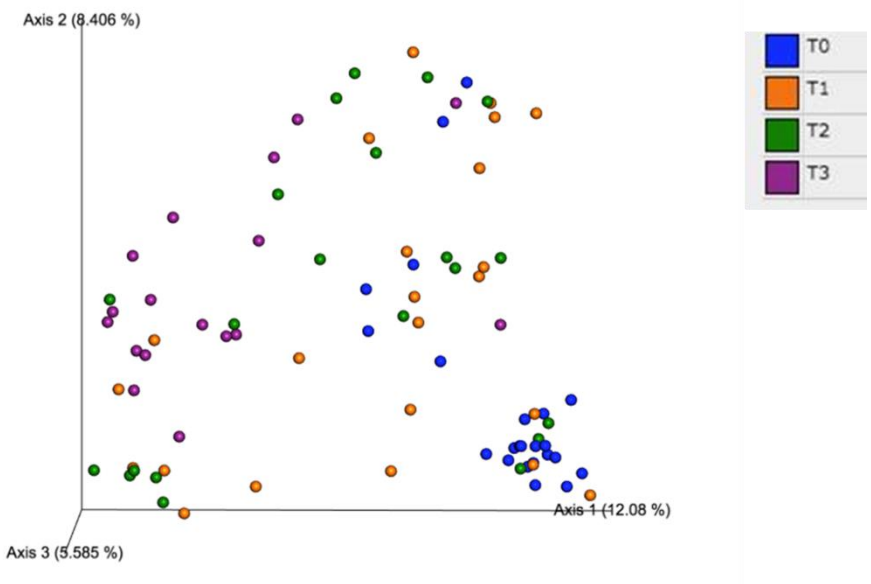
Alpha diversity increased over time, as shown in **Fig. 4A** and **4B**. The calculation of observed OTU index showed significantly higher values at T3 with respect to T1 and T2 (**Fig. 4A**); the same results were also evidenced for Shannon index, in addition to other significant differences (T0 vs T2 $p<0.05$; T0 vs T3 $p<0.05$) (**Fig. 4B**). Therefore, the increase of alpha diversity occurred after 30 days, whereas Morowitz et al. (2011) observed an increase after 16-21 days of life, with respect to the lower values detected at 10-13 days from birth, after an antibiotic treatment in the first 7 days of life. However, our study considered 23 prematures, showing high intervariability and, most likely, different nursing treatments. In this regard, a further calculation of Shannon index was performed considering the different duration of the perinatal antibiotic therapy (a: ≤72h; b :> 72h) conducted on newborns (**Fig. 4C**). No significant differences were evidenced between the two treatments. The trend in the Shannon index of the group receiving the shorter treatment (a) was fluctuating with respect to that of the group subjected to the longer one (b), which increased with time. Therefore, the prolonged antibiotic therapy at birth controlled the development of the microbiota determining a slow and gradual increase of its biodiversity.

Fig. 4. Alpha diversity calculated with Observed OTU index over time (A). Alpha diversity calculated with Shannon index over time (B). Alpha diversity calculated with Shannon index in the groups receiving the short (a: ≤72h) or prolonged (b: >72h) perinatal antibiotic treatment (C). "*" indicates p<0.05



The alpha diversity results are in accordance with the beta diversity ones based on the PCoA (Bray-Curtis distance), which showed the dispersion of samples over time (**Fig. 5**). It is possible to observe a division between most of the T0 samples and the other groups. This trend was confirmed by the ANOSIM test that evidenced significant differences between T0 samples and the other groups (T0 vs T1 $p<0.05$, T2, T3 $p<0.05$). These results showed that the gut microbiota of prematures within the first 90 days of life continually evolves and the increase of the beta-diversity is significant already at 15 days of life.

Fig. 5. Beta diversity (Bray-Curtis distance) over time illustrated with PCoA.



qPCR data

Absolute quantifications of the intestinal microbial groups analysed increased over time, as expected, showing also significant differences, especially in enterobacteriaceae and total bacteria counts (**Fig. 6C** and **6E**). These results evidenced the dynamism of gut microbiota also in infants born prematurely, although they are subjected to treatments and nursing procedures that can slow down their microbial community development.

No substantial differences were detected between the two antibiotic treatments investigated (a: ≤72h; b: >72h), but significant variations over time were found within each antibiotic therapy instead, except for *B. fragilis* group (**Fig. 6F, 6G, 6H, 6I, 6L**), demonstrating that the effects of the time impacted more than the treatment on the microbial groups analysed. However, the box plots showed more heterogeneous values for the shorter treatment (a: ≤72h), with respect to the longer one (b: >72h), for which the shorter box plots evidenced homogeneous values. Thus, the longer treatment may tend to control the development of certain bacterial groups, which resulted less affected by the shorter therapy. This trend resulted particularly evident in *Bifidobacterium* spp. and *B. fragilis* group counts (**Fig. 6F** and **6G**). Therefore, it can be hypothesized that these bacterial groups, which are two of the more representative members of infant gut after 1 week (Di Gioia et al., 2014), may contribute in maintaining the stability of alpha diversity detected by Shannon index in the group receiving the longer treatment (b: >72h).

Fig. 6. qPCR counts (Log CFU/g faeces) of microbial groups analysed for the whole cohort of subjects over time (A, B, C, D, E) and for the subjects grouped for the shorter (a: ≤72h) or prolonged (b: >72h) perinatal antibiotic therapy (F, G, H, I, L). "*" indicates $p<0.05$

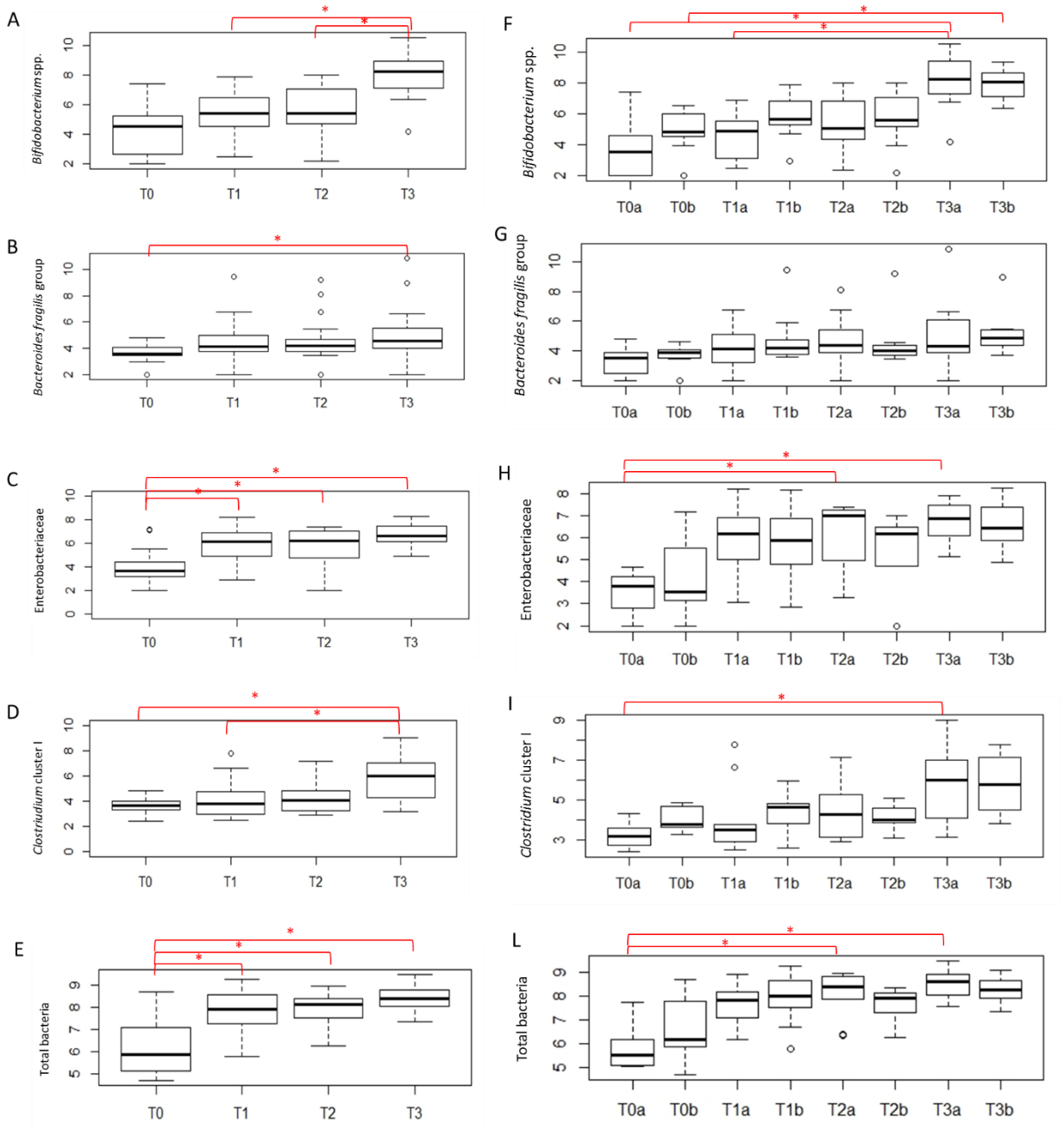
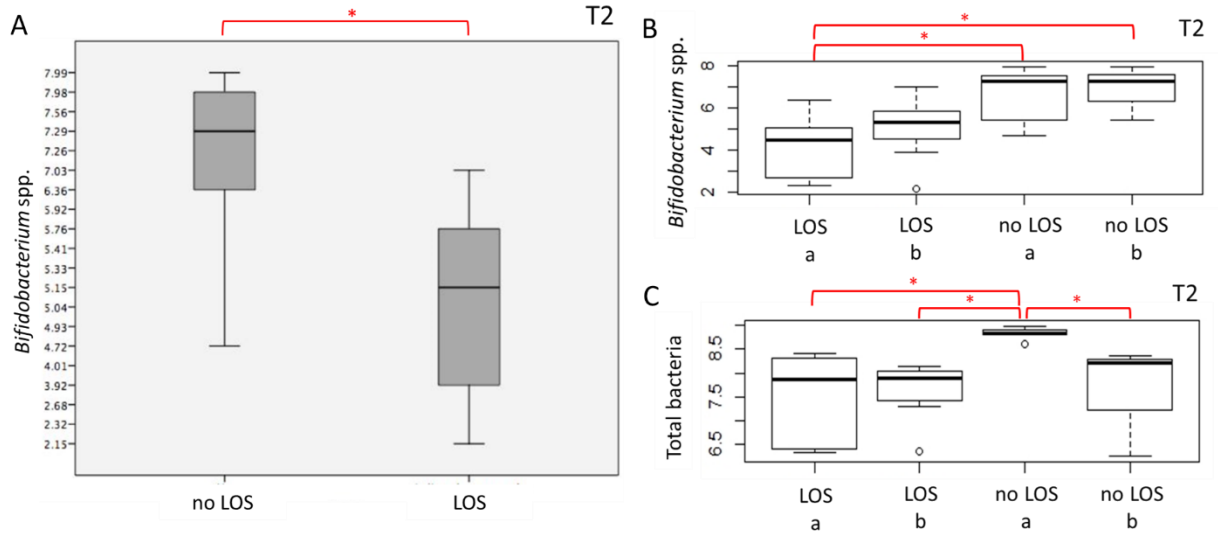


Table 3. Mean counts (Log CFU/g of faeces) of different microbial groups analysed by qPCR

	***Bifidobacterium* spp.**		***B. fragilis* group**		**Enterobacteriaceae**		***Clostridium* cluster I**		**Total bacteria**	
	≤72h	**>72h**	**≤72h**	**>72h**	**≤72h**	**>72h**	**≤72h**	**>72h**	**≤72h**	**>72h**
T0	3,72 ± 1,80	5,00 ± 1,47	3,40 ± 0,95	3,65 ± 0,76	3,94 ± 1,54	3,80 ± 1,74	3,23 ± 0,60	4,08 ± 0,60	6,00 ± 1,11	6,49 ± 1,48
T1	4,59 ± 1,55	5,82 ± 1,41	4,16 ± 1,52	4,73 ± 1,71	5,89 ± 1,60	5,76 ± 1,74	3,99 ± 1,77	4,31 ± 1,01	7,72 ± 0,85	7,94 ± 1,03
T2	5,55 ± 1,99	5,59 ± 1,69	4,68 ± 1,76	4,50 ± 1,69	6,21 ± 1,53	5,62 ± 1,48	4,62 ± 1,63	4,07 ± 0,64	8,24 ± 0,99	7,61 ± 0,76
T3	8,07 ± 1,96	7,91 ± 1,04	5,17 ± 2,67	5,26 ± 1,61	6,74 ± 0,93	6,57 ± 1,09	5,79 ± 1,97	5,79 ± 1,55	8,54 ± 0,63	8,28 ± 0,58

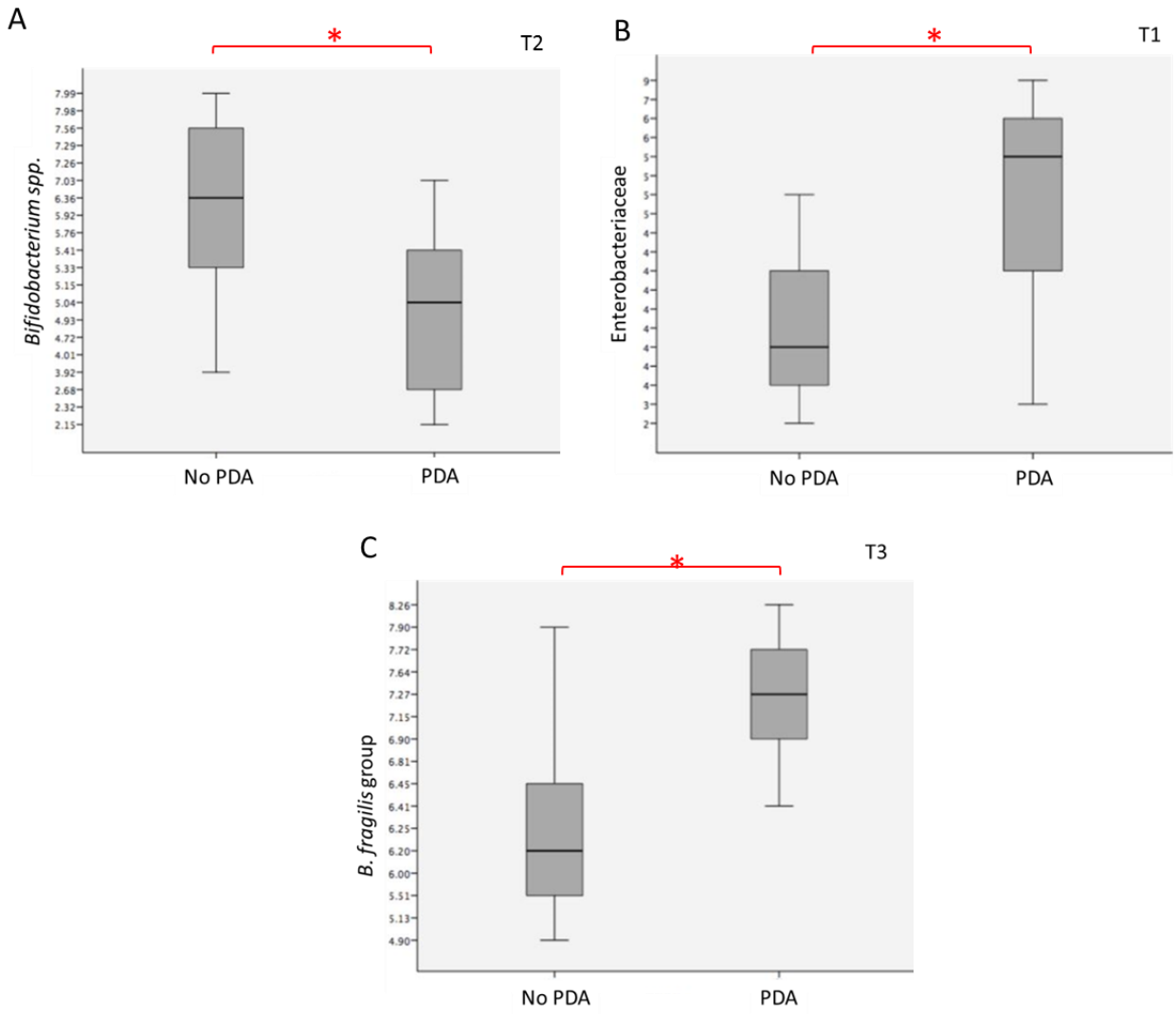
Grouping infants for the presence of LOS, at T2, those not manifesting the sepsis showed significantly higher levels of *Bifidobacterium* spp., suggesting a possible protective role against this pathology (**Fig 7A**). This was also evident in infants subgrouped for the type of antibiotic treatment, underling that the LOS had a major impact on *Bifidobacterium* spp. counts with respect to the antibiotic therapy (**Fig 7B)**. Even if not significant differences attributable to the antibiotic treatment in total bacteria were detected, within infants not manifesting LOS, those receiving the shorter antibiotic therapy (a: ≤72h) had significantly higher counts of total bacteria with respect to the group subjected to the longer one (b: >72h) (**Fig 7C**). These preliminary outcomes may favour the use of an antibiotic therapy with a duration of less than 72h.

Fig. 7. qPCR counts (Log CFU/g faeces) of *Bifidobacterium* spp. at T2 in subjects divided in those with LOS (LOS) and those without LOS (no LOS) (A), in subjects grouped for the manifestation of LOS (LOS or no LOS) and the type of perinatal antibiotic therapy (a: ≤72h, b: >72h) (B). qPCR counts (Log CFU/g faeces) of total bacteria at T2 in subjects grouped for the manifestation of LOS (LOS or no LOS) and the antibiotic therapy (a: ≤72h, b: >72h) (C). "*" indicates $p<0.05$



Also the clinical manifestation of PDA (*patent ductus arteriosus*) revealed an influence on some microbial groups (**Fig. 8**). Particularly, *Bifidobacterium* spp. counts at T2 were significantly higher in infants not manifesting the malformation, suggesting a potential protective role of this beneficial bacterial group (**Fig. 8A**). Enterobacteriaceae and *B. fragilis* counts at T1 and T3 respectively were significantly higher in PDA infants (**Fig. 8B** and **8C**). These differences may be linked to an altered blood systemic circulation and, consequently, to a reduced oxygen supply to the enteric system, affecting the normal function of the intestinal tract and the microbial population.

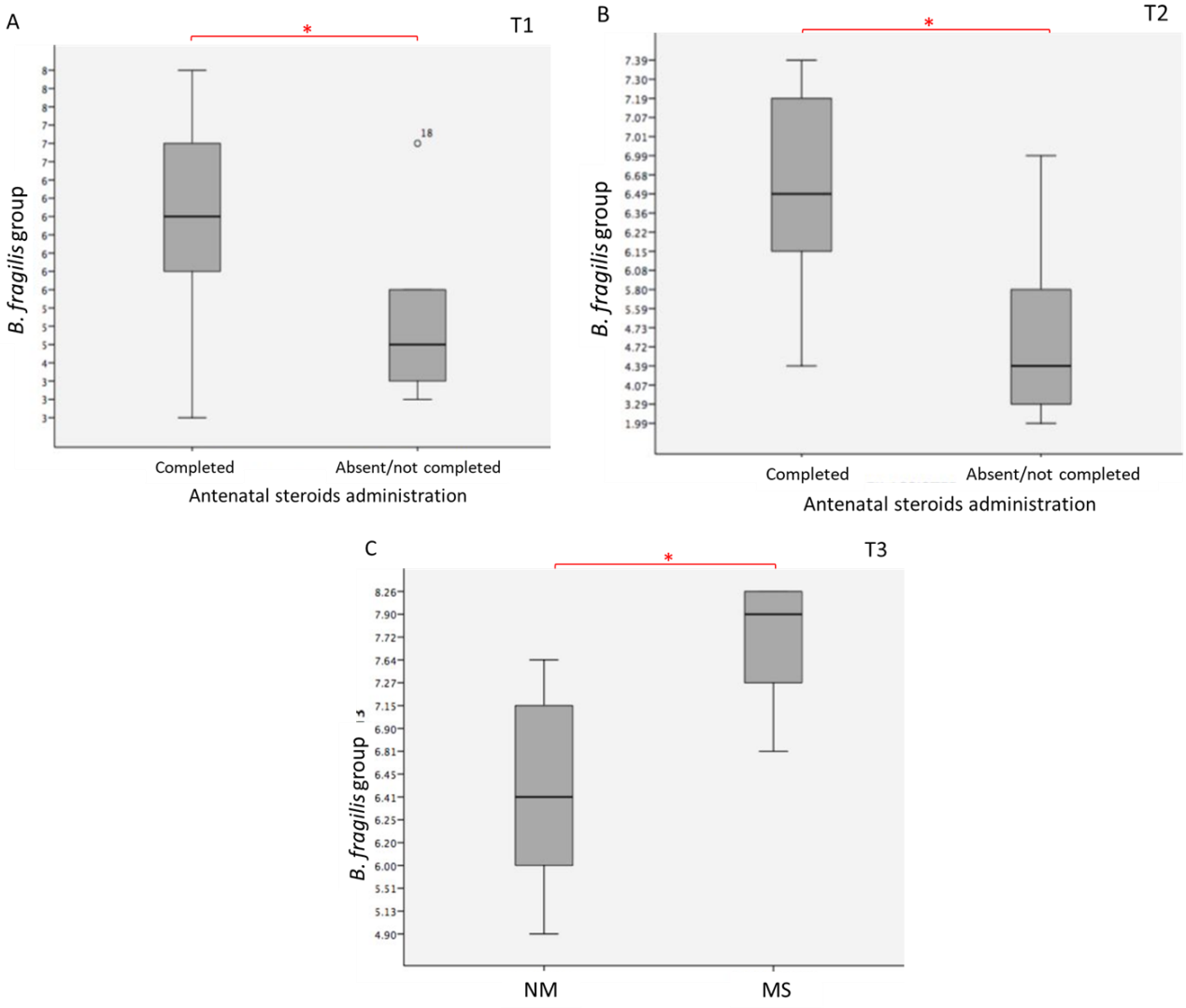
Fig. 8. qPCR counts (Log CFU/g faeces) of *Bifidobacterium* spp. at T2 (A), enterobacteriaceae at T1 (B) and *B. fragilis* group at T3 (C)in subjects manifesting PDA (PDA) and subjects not manifesting PDA (No PDA). "*" indicates p<0.05



In addition, *B. fragilis* counts showed to be influenced by respiratory clinical features as well as the genera *Escherichia* and *Clostridium*. Considering the exposure to antenatal steroids, which is well known to decrease the incidence of neonatal respiratory distress syndrome in premature infants (Liggins and Howie, 1972; Crowley, 2002), the quantification of *B. fragilis* at T1 and T2 were lower in infants for which the steroids administration was absent or not completed with respect to those for which it was completed (**Fig. 9A** and **9B**). It should be considered that infants were subjected to a complete therapy because of the gravity of the respiratory distress, on the other hand the therapy was interrupted or not carried out in infants that recovered. Moreover, regarding BPD,

B. fragilis counts were higher at T3 in MS infants with respect to NM group (**Fig. 9C**). Therefore, *B. fragilis* demonstrated to be affected by a compromised respiratory system in preterm newborns.

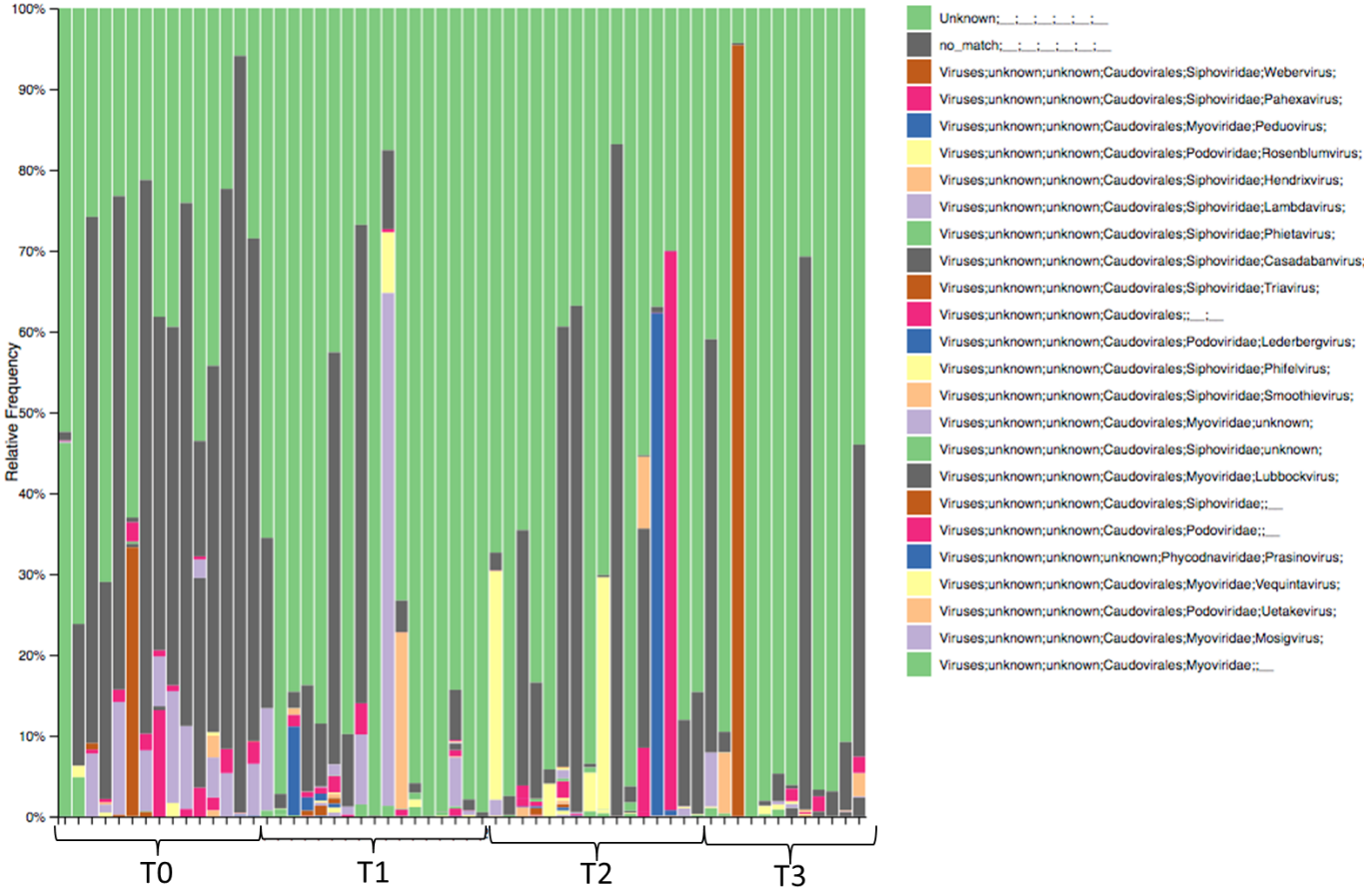
Fig. 9. qPCR counts (Log CFU/g faeces) of *B. fragilis* group at T1 (A) and T2 (B) in subjects grouped for the antenatal steroids administration (completed vs absent/not completed). qPCR counts (Log CFU/g faeces) of *B. fragilis* group at T3 in moderate/severe (MS) and absent/mild (NM) BPD (C). "*" indicates p<0.05



Intestinal bacteriophages composition

A total of 60 DNA samples were sequenced and an average of 1708 reads per sample was generated. **Fig. 10** shows a rich bacteriophages community, whose members belonged almost totally to Caudovirales order.

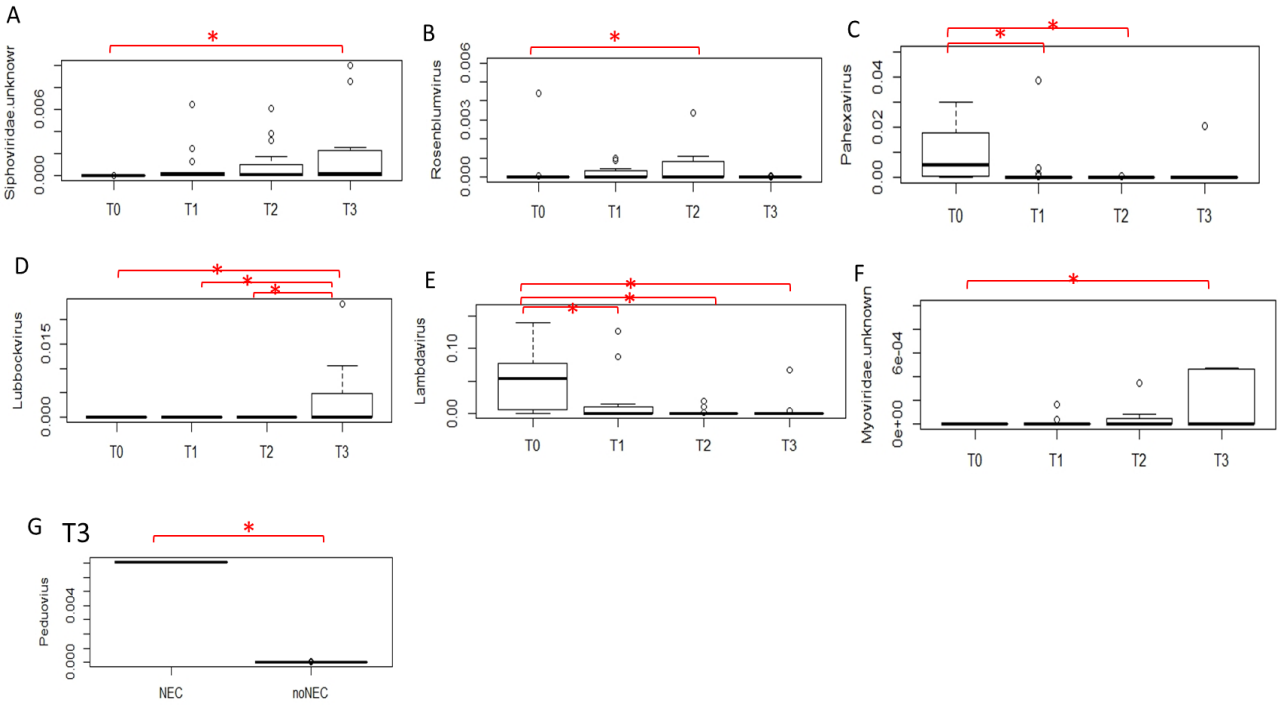
Fig. 10. Gut bacteriophages genera relative abundance in preterm infants during the study.



Some bacteriophages resulted significantly higher at T0 with respect to the other timepoints (some unknown members of Siphoviridae, *Pahexavirus* and *Lamdavirus,* **Fig. 11A, 11C** and **11E**), while some others increased over time (*Rosenblumvirus* and *Lubbockvirus*, **Fig. 11B** and **11D**). These shifts may be driven by fluctuations of the bacterial composition. As evidenced by **Fig. 11E**, *Lambdavirus* was strongly present at T0 and dropped at the following timepoints, when Enterobacteriaceae resulted dominant. It is possible that in the perinatal period the presence of *Lambdavirus* limited the increase of its host and in the following phases it was not able to control the *Escherichia* population. Nevertheless, no relevant changes in *Escherichia* relative abundance were evidenced.

Moreover, the significantly lower levels of *Peduovirus* in infants not developing NEC at T3 (**Fig. 11G**), may be linked to a protective mechanism in which this bacteriophage is able to limit bacteria responsible of the NEC onset.

Fig. 11. Siphoviridae unknown genus relative abundance at different timepoints (A). *Rosenblumvirus* relative abundance at different timepoints (B). *Pahexavirus* relative abundance at different timepoints (C). *Lubbockvirus* relative abundance at different timepoints (D). *Lambdavirus* relative abundance at different timepoints (E). Myoviridae unknown genus relative abundance at different timepoints (F). *Peduovirus* relative abundance at T3 in infants manifesting NEC (NEC) and infants not manifesting NEC (noNEC) (G). "*" indicates p<0.05



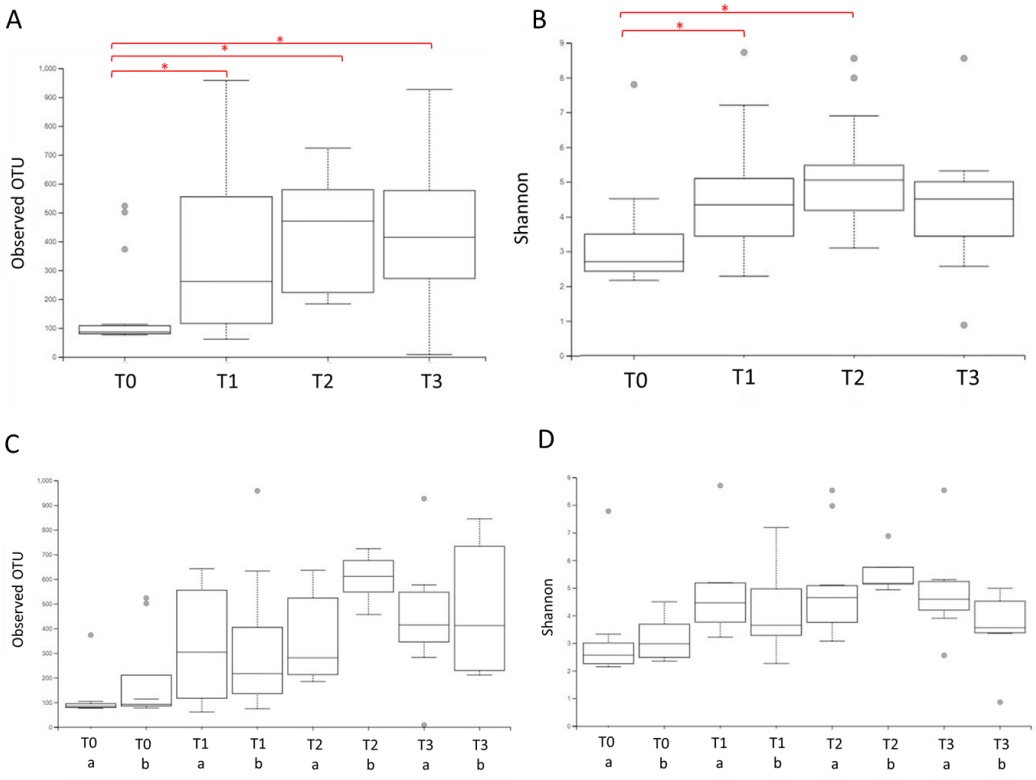
Alpha diversity increased over time and decreased in the T3 group, considering both the number of OTU (observed OTU index) and the richness and abundance of species (Shannon index) (**Fig. 11)**. Particularly, the calculation of observed OTU index showed very low values in the T0 group, which significantly differed from T1, T2 and T3 ($p<0.05$) (**Fig. 11A**). This was confirmed by the calculation of the Shannon index (T0 vs T1 $p<0.05$; T0 vs T2 $p<0.05$) (**Fig. 11B**). Some studies performed on term healthy infants showed a high phage diversity in the perinatal period that was found to decrease in the first two years of life (Bertbrait et al., 2008; Lim et al., 2015). However, it is important to point out that these studies were conducted on healthy infants and the literature on bacteriophages community in prematures is still too scarce. The prematurity may reduce, therefore, the biodiversity of bacteriophages in the perinatal period, which tends to be higher in full-term infants.

The alpha diversity of bacteriophages and bacteria followed the same trend increasing until T2; at T3 they diverged: bacterial biodiversity continued to increase and bacteriophages' one decreased. The period studied is crucial for the development of the gut microbiota and the first 30 days of life in prematures can correspond to a settlement phase for both bacteria and bacteriophages, whose interplay resulted in an increase of the whole microbiota diversity. Living as "predator" of bacteria, bacteriophages are able to control the fluctuation of bacteria, therefore their decrease in biodiversity can facilitate the development of different bacterial groups, leading to an increase of bacterial biodiversity, as shown in these preliminary results.

Although fluctuating trends were observed for both perinatal antibiotic treatments (a: ≤72h; b: > 72h), no statistical differences were detected for alpha diversity (**Fig. 11C** and **11D**).

Bacteriophages alpha diversity trend proceeded in the opposite way compared to bacterial one: the group receiving the longer treatment (b) seemed to fluctuate more with respect to the group subjected to the shorter one (a). Therefore, considering the necessity of bacteriophages to live at expense of bacteria, the perinatal antibiotic treatments may inversely influence bacteriophages alpha diversity with respect to the bacterial one.

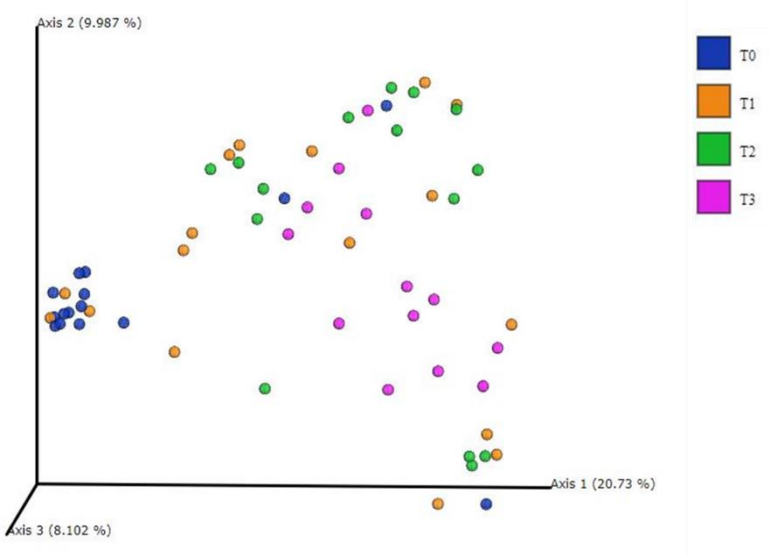
Fig. 11. Alpha diversity calculated with Observed OTU index over time (A). Alpha diversity calculated with Shannon index over time (B). Alpha diversity calculated with Observed OTU index in the groups receiving the short (a: ≤72h) or prolonged (b: >72h) perinatal antibiotic treatment (C). Alpha diversity calculated with Shannon index in the groups receiving the short (a: ≤72h) or prolonged (b: >72h) perinatal antibiotic treatment (D). "*" indicates p<0.05



Regarding the beta diversity, the PCoA (Bray-Curtis distance) showed the same separation over time detected for bacteria (**Fig. 12**). This trend was confirmed by the ANOSIM test that evidenced significant differences between T0 and T2 and T3 samples (p<0.05).

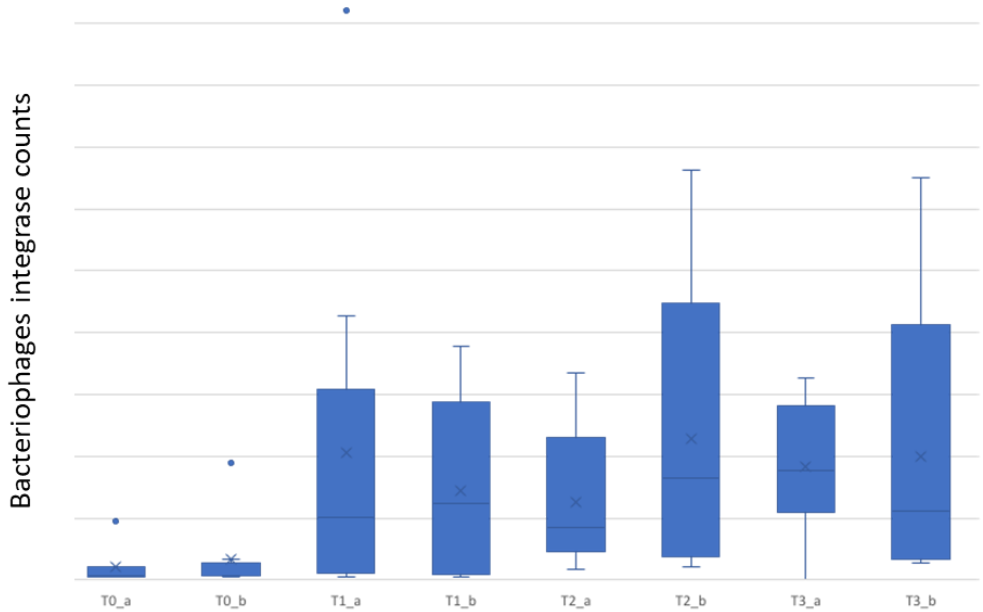
These results pointed out that, as for bacteria, the biodiversity of bacteriophages tends to increase in prematures and in the very first hours of life was reduced and markedly different with respect to that at 15, 30 and 90 days of life.

Fig. 12. Beta diversity (Bray-Curtis distance) over time illustrated with PCoA.



Bacteriophages integrase counts were also performed based on the phages sequences obtained. Differences between the two perinatal antibiotic treatments, even not statistically relevant, were detected at T2 and T3 (**Fig. 13**). Particularly, at T3, in which the infant gut microbiota likely reaches a more stable assessment, the integrase counts resulted higher in the group receiving the shorter therapy (a). This result can be explained as an effect of the shorter antibiotic therapy on bacteriophages that, having integrases, did not promote the death of their host; on the other hand, the longer therapy (b) resulting in lower integrase counts may have promoted the development of lytic bacteriophages, which, damaging their hosts, could have contributed to an instability condition of gut microbiota structure. These data may support the use of an antibiotic therapy with a duration of less than 72h.

Fig. 13. Gut bacteriophages integrase counts at different timepoints in infants grouped for the perinatal antibiotic therapy: shorter (a: ≤72h) and prolonged (b: > 72h).



Conclusions

These data evidenced a clear shift in bacterial composition in the first 3 months of life of preterm infants, with the predominance of Enterobacteriaceae in the first month. Changes in bacteriophages composition were evidenced as well and Caudovirales members prevailed.

The evolution of microbiota composition was highlighted by the increase of biodiversity in both bacteria and bacteriophages over time. The increasing of alpha diversity diverged at 3 months, when bacteriophages trend decreased, suggesting the possible development of bacteria that cannot be infected by bacteriophages.

Gut microbiota resulted strongly influenced by the clinical picture of preterm infants. The use of antenatal steroids and BPD evidenced the possible connection between a condition of respiratory distress and a gut bacterial environment characterized by a reduction of beneficial bacteria and an increase of potentially harmful bacteria. Particularly, *B. fragilis* counts were influenced by the respiratory condition. In addition, *Bifidobacterium* spp. counts were significantly higher in preterm infants not developing LOS and PDA suggesting a possible protective role of bifidobacteria against these diseases. The bacteriophage *Peduovirus*, resulting higher in preterm infants not developing NEC, may impede the survivor and, therefore, the blood translocation of intestinal bacteria responsible of NEC onset.

Perinatal antibiotic treatments of different duration may influence in a divergent manner bacterial and bacteriophage biodiversity. In particular, the prolonged treatment (> 72h) tended to slow down the diversification of the bacterial community, although without a statistical relevance, especially for two of the major members of the infant gut microbiota, *Bifidobacterium* spp. and *B. fragilis* group. In addition, the bacteriophages integrase counts suggested that the shorter antibiotic treatment (≤ 72h) may select lysogenic bacteriophages to the detriment of lytic ones. This outcome, together with the more heterogeneous values of bacterial counts and the higher levels of total bacteria detected in infants that underwent the shorter antibiotic treatment, may promote the use of an antibiotic therapy at birth with a duration less than 72h in order to reduce the impact of the therapy on the correct development of gut microbiota and, consequently, to protect the host homeostasis later in life.

Moreover, the results obtained from this study will allow to design a targeted microbial strategy that can be supplemented to integrate and ameliorate the correct establishment and development of gut microbiota in preterm infants and to guarantee to the best the maintenance of the health status later in life.

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GENERAL DISCUSSION AND CONCLUSIONS

The work performed in this thesis shows, overall, that the gut microbiota is altered in the disease/disorders considered in this study with respect to healthy subjects and that, in some cases, targeted probiotic administration can help in restoring the gut dysbiosis.

Alterations in the gut microbiota between healthy and diseased patients have been observed in two diseases targeting different organ systems, such as coeliac disease and ALS.

Paper 3 and **4** showed a clearly separation of gut microbiota profiles between healthy and coeliac subjects, mostly attributable to Verrucomicrobia, Parcubacteria and some other unknown bacterial phyla. The absence of a severe dysbiosis in coeliac patients was likely associated to the adherence to a gluten free diet. The impact of the diet on gut microbiota has been confirmed by the scarce abundance of Euryarchaeota and related families in coeliac children respect to healthy subjects; this interesting outcome, still not investigated in the literature, may be linked to the adherence to a diet that is poor of polysaccharides (Samuel and Gordon, 2006).

Paper 6 is quite *avant-garde* focusing on a neurological disorder whose pathogenesis is still obscure and whose relationship with the intestinal microbiota has been scarcely investigated in humans. The work showed the interesting high abundance of Cyanobacteria in ALS patients with respect to controls, re-opening a window for a hypothesis formulated in 2000s and then shelved. In order to better clarify the role of Cyanobacteria in ALS, a further investigation should be conducted, such as the absolute quantification of Cyanobacteria and neurotoxin producing bacteria by qPCR in healthy controls' and patients' DNA samples collected at the baseline and during the follow-up to monitor their fluctuation in the course of disease progression.

The dissertation also explored **the effect of probiotic administration** in some diseases.

Paper 1 allowed the conclusion that the *B. breve* species have the potential for a targeted use in a large spectrum diseases both in paediatric subjects and in adults. This is supported not only by the positive results obtained in the trials described in the literature but also by the safe use of this species, which has the QPS status.

In this context, three works focused on the effects of *B. breve* based formulation (B632 and BR03) in preventing or treating paediatric disorders were carried out.

A three-months treatment with the two *B. breve* strains in healthy breast-fed newborns (**Paper 2**) helped to prevent functional gastrointestinal disorders, in particular reducing daily vomit frequency, daily evacuation over time and improving the stool consistency. Moreover, a significant reduction of *B. fragilis* in the bottle-fed group receiving the probiotic formulation was observed. This study also outlined that children born by caesarean section, who have an high risk to develop obesity later in life, showed a lower catch-up growth in weight. This allows the speculation that the administered formulation has a role in reducing the risk of metabolic disturbances later in life.

This thesis also evidenced the positive effects deriving from *B. breve* strains supplementation to obese children (**Paper 5)** in improving glyco-insulinemic profile and, although not statistically significant, in body fat composition. A metagenomic analysis will integrate the results obtained

providing further information about the impact of the probiotic supplementation on bacterial ecosystem and the entire intestinal bacterial community.

Moreover, the PhD work allows to reach the conclusion that a three-month administration of *B. breve* strains to coeliac children helps in restoring the healthy percentage of main microbial components. **Paper 3** showed that probiotics supplementation can influence not only on the bifidobacteria population but the administered strains can act as "trigger" elements for the increase of Firmicutes and the restoration of the physiological Firmicutes/Bacteroidetes ratio. The Firmicutes family mostly influenced was Lactobacillaceae that reached almost the values characterizing the healthy subjects. The increase of Firmicutes evidenced in **Paper 4** was correlated to a decrease of pro-inflammatory TNF-α, confirming the role of gut microbiota in modulating the host's inflammatory condition. This study also reported a new evidence about the potential anti-inflammatory role of Synergistetes, which negatively correlated with pro-inflammatory acetic acid in coeliac children after the probiotic administration.

The six-months probiotic treatment with a mixture of *Lactobacillus* strains of ALS patients (**Paper 6**) influenced in different ways the gut microbial composition; however, none of the probiotic interventions brought the biodiversity of intestinal microbiota of patients closer to that of healthy subjects. Although the suggested probiotic formulation only influenced few microbial groups not affecting the main clinical parameters measured in ALS, it was possible to observe some changes in the gut microbial environment in relation to the diseases progression, confirming the special role of gut microbiota also in pathologies involving districts relatively far from the intestinal tract. This is the first study that clearly shows the modifications of gut microbiota composition in ALS patients by applying novel and rigorous methodologies. This approach based on microorganisms' administration can be further improved with the design of a new formulation and applied in larger clinical studies in order to characterize the microbiota changes as a novel biomarker of the disease.

During the last six-months of my PhD program, I approached the **new and poorly explored field of bacteriophages** in the gut. In this regard, **Paper 7**, elucidating not only the intestinal bacterial composition but also the bacteriophages' one in the gut of preterm newborns, provided some preliminary outcomes also in relation to the antibiotic therapy that is a must for prematures. The increase of biodiversity in both these two microbial communities from birth until one month of life reflected the dynamism of the gut microbiota in the first period of life also in preterm infants. The following divergent trend observed at three months (increased bacterial biodiversity and decreased bacteriophage's one) can be ascribed to the selection of a more stable microbiota in which the predator-prey relationship is at detriment of the bacteriophages diversity. These information, supported by clinical evidences, can pose the basis for the development of a targeted probiotic formulation that can be administered together with antibiotics, which are quite essential for prematures, driving the establishment of a "healthy" microbiobiota community able to protect the host's homeostasis also later in life.

I would like to conclude this dissertation with a general consideration. Being the gut microbiota a complex ecosystem, it possess a certain resilience (Sommer et al. 2017). This property determines whether a particular perturbation will permanently shift its stable state or whether it will return to its initial homeostatic state after a perturbation. The dysbiosis can occur when resilience of the original

community fails and this condition can lead to the acquisition of an unhealthy microbiota that can become resilient in turn. In this regard, a probiotic administration is necessary:

1. to generally maintain the resilience of a healthy microbiota in order to face any perturbation;
2. to protect the microbiota's resilience in a vulnerable period, such as during the first years of life when the microbiota is establishing, or during a period of stress or reduced activity of the immune system;
3. to contrast the establishment of a unhealthy resilient microbiota;
4. to reduce at the least the consequences associated to a permanently altered microbiota

This thesis confirmed that the gut microbiota represents a key determinant of the overall health status and disease susceptibility of humans. The use of microorganisms can be an effective strategy to reduce symptoms related to the disease and to re-establish the beneficial microbiota in the gut.

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