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The role of bifidobacteria in newborn health and the intestinal microbial balance

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The role of bifidobacteria in newborn health and the intestinal microbial balance

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Abstract

Gut microbial acquisition during the early stage of life is an extremely important event since it affects the health status of the host. In this contest the healthy properties of the genus *Bifidobacterium* have a central function in newborns.

The aim of this thesis was to explore the dynamics of the gut microbial colonization in newborns and to suggest possible strategies to maintain or restore a correct balance of gut bacterial population in infants. The first step of this work was to review the most recent studies on the use of probiotics and prebiotics in infants. Secondly, in order to prevent or treat intestinal disorders that may affect newborns, the capability of selected *Bifidobacterium* strains to reduce the amount of *Enterobacteriaceae* and against the infant pathogen *Streptococcus agalactiae* was evaluated *in vitro*. Furthermore, the ability of several commercial fibers to stimulate selectively the growth of bifidobacterial strains was checked. Finally, the gut microbial composition in the early stage of life in response to the intrapartum antibiotic prophylaxis (IAP) against group B *Streptococcus* was studied using q-PCR, DGGE and next generation sequencing.

The results globally showed that *Bifidobacterium breve* B632 strain is the best candidate for the use in a synbiotic product coupled to a mixture of two selected prebiotic fibers (galactooligosaccharides and fructooligosaccharides) for gastrointestinal disorders in infants. Moreover, the early gut microbial composition was affected by IAP treatment with infants showing lower counts of *Bifidobacterium* spp. and *Bacteroides* spp. coupled to a decrement of biodiversity of bacteria, compared to control infants. These studies have shown that IAP could affect the early intestinal balance in infants and they have paved the way to the definition of new strategies alternative to antibiotic treatment to control GBS infection in pregnant women.

Key words: Gut microbiota, *Bifidobacterium*, probiotics, prebiotics, q-PCR, DGGE, next generation sequencing, GBS infection

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Background

1. Early gut microbiota

1.1 Acquisition and microbial composition

The human gut microbiota consists of a complex population of microorganisms acquired during the early stages of life. As summarized by Claesson et al. (2011), microbial composition of the gut is subjected to major changes during three different time-points in the life of a human being:

1) the neonatal period; after birth the bacterial colonization of the gastrointestinal tract (GIT) starts and it is influenced by different factors such as diet, mode of delivery, prematurity, etc;
2) the weaning period; gradual introduction of solid foods, approximately at 4-6 months of life, and consequent exposition to complex nutrients which drive to an adult-like microbiota;
3) the elderly period; it occurs at around sixty years and is characterized by physiological modifications of the gut and consequently profound changes in the microbiota composition.

At the end of the second stage, when the mature microbiota is established, the large intestine is colonized by 300-500 bacterial species which reach the number of ~ 10^{12} – 10^{15} CFU/g of lumen content.
Following birth the newborn's gut encounters a large number of microorganisms (Hansen et al. 2012), firstly from the uro-genital tract of the mother and secondly from the environment (Penders et al. 2006) even though the first microbial contamination has probably an intrauterine origin; different studies have already shown that amniotic fluid and meconium are not sterile (DiGiulio et al. 2008; Mshvildadze et al. 2010; Moles et al. 2013). At birth, the GIT of the newborns has a positive oxidation/reduction potential, consequently facultative anaerobes (*Staphylococcus* spp., *Enterobacteriaceae* and *Streptococcus* spp.) are the first microorganisms that colonize this environment (Songjinda et al. 2005). Following the first days of colonization, the development of the microbiota is influenced by a progressive oxygen consumption and the subsequent growth of strict anaerobes (*Bifidobacterium* spp., *Bacteroides* spp. and *Clostridium*

spp.) (Biasucci et al. 2010). The initial gut bacterial colonization is characterized by a low biodiversity and results unstable up to 2 years of age when it becomes similar to the adult one (Adlerberth and Wold 2009; O'Toole and Claesson 2010; Koenig et al. 2011).

1.2 Factors affecting the microbial composition

The acquisition of gut microbiota during the postnatal period is hardly connected to the development of the host's immunity and generally to the host's health. Mode of delivery, type of feeding, prematurity and antibiotic exposure are the main factors shaping early microbial composition of the gut.

1.2.1 Mode of delivery

The mode of delivery has been reported to have a great influence for the initial colonization of the GIT (Penders et al. 2006). In vaginally delivered infants the time of colonization and the microbial composition differ compared to those delivered by caesarian section (Biasucci et al. 2010). After being vaginally born the infants inherit fecal and vaginal bacteria from the mother; the vertical transmission leads to an immediate colonization of lactobacilli (Dominguez-Bello et al. 2010). On the other hand, infants born by caesarian section are exposed to the bacteria belonging to the hospital environment; these infants are characterized by a reduction and delay of the colonization of bifidobacteria and *Bacteroides* spp. and meanwhile by an increase of potentially harmful bacteria such as *Escherichia coli*, *Clostridium perfringens* and *Clostridium difficile* (Penders et al. 2006; Biasucci et al. 2010). Furthermore, the microbial richness and biodiversity of infants born by caesarian section result significantly lower compared to the ones vaginally born (Azad et al. 2013).

1.2.2 Type of feeding

The heavy influence of breastfeeding on the gut microbiota colonization during the first month of life has been established (Allen and Hector 2005; Penders et al. 2006); the World Health Organization (WHO 2001) promotes exclusive breastfeeding at least up to 6 months and then, with the introduction of solid foods, it advises to continue breastfeeding for up to 2 years of life. Important differences have been noted between the intestinal colonization of breast-fed (BF) and formula-fed (FF) infants. The increased species richness and the unstable microbial colonization have been observed for FF infants compared to BF infants who have higher levels of *Bifidobacterium* and *Lactobacillus* (Fallani et al. 2011; Azad et al. 2013; Fan et al. 2013). In

addition, differently from BF, FF infants have much higher counts of *Clostridium* spp. and facultative anaerobic bacteria such as the *E. coli* (Penders et al. 2006; Bezirtzoglou and Stavropoulou 2011). The positive imprinting of BF in shaping the gut microbiota can be explained by analyzing the nutrition characteristics of the human milk. Oligosaccharides of human milk (HMOs), described in **section 3.2.2**, represent one of the most nutritional constituent of the milk and have an important prebiotic action towards the growth of specific bacterial species, such as *Bifidobacterium* (Coppa and Gabrielli, 2008; Bode 2009). In addition, human milk contains immune-modulator factors, such as Igs, and antimicrobial molecules, such as lysozyme and lactoferrin (Field 2005).

It is well documented that human milk has a lower buffering capacity compared to formula milk. This difference, which leads to a reduction of pH in the colon, encourages the growth of acid tolerant species such as bifidobacteria and lactobacilli while results inhibitory to other bacteria (Tham et al. 2011).

Moreover, the positive effect of BF is also due to the presence of bacteria in human milk, mainly lactic acid bacteria such as streptococci, lactobacilli, enterococci, as well as bifidobacteria (Kagnoff 2007; Arboleya et al. 2011; Jost et al. 2012).

1.2.3 Prematurity and antibiotic exposure

The gut microbiota composition of premature infants (born before 37 weeks of gestation) differs from that of full term infants. Immaturity of the immune response, exposure to broad-spectrum antibiotics, delay in feeding, can influence the bacterial colonization in preterm infants (Mai et al. 2013). As a result, up to the first three months of life, the gut microbiota has low diversity of taxa, reduced proportions of strict anaerobes and increased colonization by facultative anaerobic bacteria, such as enterobacteria and enterococci (Magne et al. 2008; Arboleya et al. 2012). In addition it has also been observed that the counts of *Bifidobacterium* are reduced and generally delayed during the first days of life (Fanaro et al. 2003; Westerbeek et al. 2006). Independent studies have suggested a connection between the altered microbial composition in preterm infants and the increased risk of systemic inflammatory response syndrome, sepsis and gastrointestinal disorders such as necrotizing enterocolitis (NEC) (Lin et al. 2008; Bolker et al. 2009; Sherman 2010; Barrett et al. 2013). Usually a premature birth is associated with hospitalization and often requires the use of an antibiotic therapy. The impact of the antibiotic exposure on the intestinal microbiota leads to the reduction of the main anaerobic bacteria with, as a consequence, the overgrowth of enterococci and *Enterobacteriaceae* (Fouhy et al. 2012). Another important impact of antibiotic administration is on the growth of lactobacilli and

bifidobacteria resulting in decreased number and, in some cases, absence (Bennet et al. 2002; Mangin et al. 2010).

1.3 Health-promoting functions of the gut microbiota

The gut microbiota, defined as a microbial organ inside the human host, plays an important role in many aspects of human health and diseases (Guarner 2006).

Even though some of the bacteria in the gut are pathogens or potential pathogens and can be a source of infection, the majority of bacteria are in a symbiotic relationship with the GIT exerting beneficial effects on the host (Hooper 2004).

To better understand the anatomic characteristics and physiological functions that are associated with the the gut bacteria, studies on germ-free animal models have been carried out (Wostmann 1996). Germ-free animals exhibit an underdeveloped immune system, indicating that gut bacteria influence the immune system's development and balance compared with conventional animals (Strauch et al. 2005). Studies also indicate that the microbiota has an important effect on the proper organ development (heart, lung, and liver), neural development and function, cardiac system, intestinal homeostasis (Guarner et al. 2006; Cryan and Dinan 2012). The main functions of the microbiota on the host can be referred to the metabolic, protective, trophic and immune stimulation functions which will be analyzed in this chapter.

1.3.1 Metabolic function

The metabolic functions of the intestinal bacteria consist in the fermentation of undigested fibers and oligosaccharides in the colon producing short chain fatty acids (SCFA), propionate and butyrate which can be absorbed by the intestinal epithelial cells (60–70 % of their energy requirements derives from bacterial fermentation products) (Montalto et al. 2009; Le Roy et al. 2013).

SCFAs influence the metabolism of the colon allowing the epithelial cells to salvage energy, moreover it has been observed that some SCFAs, such as acetic acid, can modulate cell turnover reducing the risk of development of inflammatory disease (Hooper 2004; Comalada et al. 2006). In addition the SCFAs enhance selectively the growth of certain bacteria, such as lactobacilli and bifidobacteria, which represent an important source of vitamins (including folates, biotin, vitamin K) and favour the absorption of ions (Ca, Mg, Fe) in the caecum (Camilo et al. 1996; Bäckhed et al. 2004; O'Hara and Shanahan 2006).

1.3.2 Protective function

The intestinal mucosal surface forms a barrier between the lumen, which comes in direct contact with digested food and non-sterile internal environment, and the sterile body (Duerr and Hornef 2012). Consequently the epithelium of the mucosal surface may be subjected to the microbial attack by microorganisms. In this contest, the gut microbiota is able to prevent invasion by enteric pathogens through different mechanisms including secretion of antimicrobial factors (such as bacteriocins), displacement, competition for nutrients and attachment to ecological sites and stimulation of the mucosal immune system of the host (Lievin 2000; Jankowska et al. 2008).

1.3.3 Trophic and immune-stimulatory function

Bacteria have a direct impact on the morphology of the gut, the enteric microbiota can control proliferation and differentiation of epithelial cells. Compared to germ-free mice, those colonized at birth have an increased cellular turnover in the intestinal crypts. In addition components of the microbiota modify the differentiation programs of intestinal epithelial lineages during morphogenesis (Hooper 2004).

The enteric bacteria play an important role in the development of the gut associated lymphoid tissue (GALT) which constitutes a major part of the mucosal immune system (Hooper 2004; Mason et al. 2008). The GALT is organized in an inductive site of immune responses, comprehensive of lymphoid follicles (Peyer's patches), and effector sites, constituted by the lamina propria and surface epithelium (Brandtzaeg and Pabst 2004). The follicle-associated epithelium contains M cells that gather small particles and transport them from the gut into the organized lymphoid tissue (Neutra et al. 2001).

In germ-free mice, several studies have shown that the intestinal immune system is underdeveloped, showing hypoplastic Peyer's patches, reduction of circulating CD4 or plasma cells (Hooper et al. 2012). The immune system is able to discriminate self and non-self or pathogenic and beneficial bacteria mainly thought the pattern recognition receptor systems (PRR), which belong to the family of Toll-like receptors (TLRs) (Vinderola et al. 2005). These receptors, which are expressed by macrophages, dendritic cells, endothelial and epithelial cells, recognize the PPR ligand, such as peptidoglican, lipopolisaccharides, lipoteichoic acids (Medzhitov 2001; Takeda and Akira 2005). It has been also demonstrated that some commensal bacteria, such as LAB and bifidobacteria, present the PPR ligand and are able to send immune signals to the intestinal cells to fight pathogens (Kelly et al. 2005).

In response to bacterial infection, intestinal epithelial cells can release cytokines such as IL-6, IL-8, tumor necrosis factor alpha (TNF-α) and gamma interferon (INF-γ) (Perdigón et al. 2002;

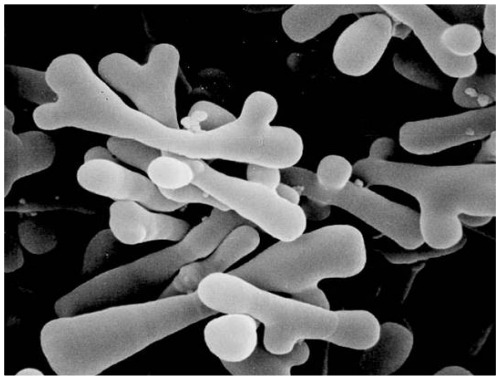
Tanoue et al. 2008). On the other hand, the infant's intestinal cells are immature, the secretion of cytokines, induced by pathogenic bacteria, may be over-expressed during the inflammatory response and thus lead to the development of gut-related pathologies, such as IBD and necrotizing enterocolitis (NEC) (Edelson et al. 1999). In this context the interaction between some commensal strains and intestinal cells can down-regulate the spontaneous release of cytokines, such as TNF-α playing an important role in the innate immune response induced by probiotics (Borruel 2002; Vinderola et al. 2005; Cencic and Langerholc 2010).

Moreover, several studies have evidenced stimulatory effect of the microbiota towards the T-cells with the secretion of non-inflammatory IgA (Cerutti and Rescigno 2008; Dogi et al. 2008).

2. The genus *Bifidobacterium*

2.1 Taxonomy and ecologic distribution

Tiesser was the first to discover bifidobacteria, which were characterised by a Y-shaped morphology in infant feces. The first isolate was referred to as *Bacillus bifidus communis* (Tiesser 1899). For a long time, bifidobacteria were included in the *Lactobacillus* genus. Only in 1974 they were officially classified in the genus *Bifidobacterium* in the Bergey's Manual of Determinative Bacteriology.



Domain:	**Bacteria**
Phylum:	**Actinobacteria**
Class:	**Actinobacteria**
Order:	**Bifidobacteriales**
Family:	***Bifidobacteriaceae***
Genus:	***Bifidobacterium***

The genus *Bifidobacterium*, according to Taxonomic Outline of the Prokaryotes, belongs to the phylum *Actinobacteria*, class *Actinobacteria*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. Other genera belonging to this family are: *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia* and *Scardovia* (Biavati and Mattarelli 2012).

Bifidobacteria have been mainly isolated from the intestine of human and other warm-blooded mammals even though a large number of species are usually detached in different ecological niches such as the human vagina and oral cavity, the animal and insect intestine, and sewage.

At present, the genus *Bifidobacterium* includes 41 species which are grouped in the table 1 according to the natural habitat.

Table 1 Species of the genus *Bifidobacterium* and their ecological origins.

Species	Habitat*
B. angulatum	Human feces
B. gallicum	Human feces
B. scardovii	Human feces
B. animalis	Animal feces
B. adolescentis	Intestine of adult
B. catenulatum	Intestine of adult and infant
B. longum	Intestine of adult and infant
B. bifidum	Intestine of infant
B. breve	Intestine of infant
B. pseudocatenulatum	Intestine of infant
B. callitrichos	Feces of marmoset
B. reuteri	Feces of marmoset
B. biavatii	Feces of red-handed tamarin
B. saguini	Feces of red-handed tamarin
B. stellenboschense	Feces of red-handed tamarin
B. mongoliense	Fermented milk
B. crudilactis	Raw milk
B. boum	Cattle rumen
B. merycicum	Bovine rumen
B. ruminantium	Bovine rumen
B. saeculare	Rabbit feces
B. cuniculi	Feces of rabbit
B. magnum	Feces of rabbit
B. gallinarum	Feces of chicken
B. pullorum	Feces of chicken
B. psychraerophilum	Swine feces
B. choerinum	Swine feces
B. thermophilum	Swine feces
B. pseudolongum	Bovin rumen and swine feces
B. thermacidophilum	Wastewater and feces of piglet
B. minimum	Sewage
B. subtile	Sewage
B. actinocoloniiforme	Intestine of bumblebees
B. bohemicum	Intestine of bumblebees
B. bombi	Intestine of bumblebees
B. asteroids	Hindgut of honeybee
B. coryneforme	Hindgut of honeybee
B. indicum	Hindgut of honeybee
B. dentium	Dental caries and intestine of infant
B. inopinatum	Dental caries
B. tsurumiense	Dental caries

*Habitat from which the species were originally isolated

2.2 Bifidobacterial composition in the infant gut

The first studies on the bifidobacterial composition in infants were performed through culture-based and DNA–DNA hybridization techniques (Benno et al. 1984; Mitsuoka 1984; Biavati et al. 1986). It was found that the total bifidobacterial count was higher in breastfed infants than in formula-fed ones. The most represented species in breast-fed and formula-fed infants were *B. infantis* (at present classified as *B. longum* subsp. *infantis*), *B. breve*, *B. longum* (at present classified as *B. longum* subsp. *longum*), and *B. bifidum*. *B. pseudocatenulatum* and *B. catenulatum* and *B. dentium* were also present although in a lower number not being the dominant species.

In the last twenty years with the development of molecular techniques, the way of performing microbial ecology studies has completely revolutionized.

In the study of Klaassens et al. (2009), quantitative PCR (qPCR) has basically confirmed what traditional plate counts had already stated, in addition *B. adolescentis* was found exclusively in formula-fed infants and *B. animalis* and *B. dentium* were not detectable in the samples irrespective to the feeding method.

It is well known that high levels of the bifidobacteria in the early-intestinal microbiota are associated to an healthy-status of the infant (Hart et al. 2004). The microbiota of infants, being less stable compared to that of adults, shows usually a large variation in composition and relative abundance of the *Bifidobacterium* species (Penders et al. 2006; Claesson et al. 2011). Several studies reported an adult-like bifidobacteria composition in the formula-fed and allergic infants which resulted colonized by higher level of *B. catenulatum* and *B. adolescentis* (He 2001; Haarman and Knol 2005).

According to a recent study based on 16S rRNA sequencing (Turroni et al. 2012a), the dominant bifidobacterial species detected in the fecal samples of healthy infants were *B. longum* and *B. bifidum*. *B. breve*, *B. adolescentis* and *B. pseudocatenulatum* were also present although in a lower proportion. Despite the fact that the literature shows highly variable results on the frequency and abundance of *Bifidobacterium* in the gut microbiota, it widely evidenced that the "bifidobacterial core" characterizing the infant's gut consists of *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum* and *B. bifidum*. In addition the species *B. catenulatum, B. pseudocatenulatum, B. dentium* and *B. adolescentis* can also be detected although their presence in closely linked to many factors affecting the early microbial composition (see **section 1.2**).

2.3 Physiologic and metabolic properties

Bifidobacteria are Gram-positive, polymorphic branched rods (Y or V-shaped) that occur singly, in chains or in clumps, non-spore forming, non-motile, non-filamentous, catalase negative (with some exceptions). Bifidobacteria are anaerobic microorganisms. However, the sensitivity to oxygen is a species- and even strain-specific characteristic. Optimum temperature for growth is 37–41°C, some changes occur depending upon the habitat of origin, i.e., the growth at 45°C seems to discriminate between animal and human strains, since most of the animal but not the human strains are able to grow at this temperature (Gavini et al. 1991; Dong et al. 2000). Bifidobacteria are acid tolerant but they are not acidophilic microorganisms; optimum pH for growth is between 6.5 and 7.0.

Their genome guanine and cytosine (G+C) content of DNA is quite high (from 42 mol% to 62 mol%), with differences among species (Biavati and Mattarelli 2012).

Obtaining energy from the oxidation of reduced organic compounds, bifidobacteria are chemoorganotrophs with a saccharoclastic metabolism; they produce acid but not gas from a variety of carbohydrates.

Bifidobacterium spp. produce lactic and acetic acid from glucose. The global equation is:

$$\mathbf{2\ glucose + 5\ ADP + 5\ P_i \rightarrow 3\ acetate + 2\ lactate + 5\ ATP}$$

The key enzyme of this metabolic pathway (fructose-6-phosphate shunt or bifidus shunt) is fructose-6-phosphate-phosphoketolase, which is considered a taxonomic character for the identification at the genus level (Biavati and Mattarelli 2012).

To better understand the bifidobacteria adaptation to specific ecological niches, such as the human intestine, the complete genome of some strains has been sequenced. Based on such analyses, different species and more specifically different strains are able to express factors involved in carbohydrates metabolism, colonization and persistence within the gut environment. Bifidobacteria utilize complex carbohydrates obtained either from the diet or from the host that are normally undigested in the small intestine (Sánchez et al. 2013).

About 6.5% of the bifidobacteria-conserved genome codes for proteins involved in carbohydrate metabolism, such as diverse glycosyl hydrolase (GH) enzymes and sugar transporters (Bottacini et al. 2010; González-Rodríguez et al. 2013).

It has been demonstrated that *B. bifidum* PRL2010, a strain isolated from infant stool, has several genes involved in nutrient-acquisition strategy that targets host-derived glycoproteins, such as those present in mucin (Turroni et al. 2010).

Genome analyses of the strain *B. longum* NCC2705 showed an excessive number of genes, representing more than 8% of the whole genome. The total predicted proteins are associated with oligosaccharide transport and metabolism, human intestinal mucus metabolism and dietary nondigested carbohydrates breakdown (Schell et al. 2002; Falony et al. 2006; Ruiz et al. 2011).

The infant gut inhabitant *B. longum* subsp. *infantis* is highly specialized in the use of the HMOs present in breast milk. The genome of the strain ATCC15697 contains a gene cluster that encodes GH and carbohydrate transporters that are necessary for importing and metabolizing these HMOs (Sela et al. 2008).

The persistence of specific bifidobacteria in the gut is the result of a strong environmental pressure which has amplified the level and diversity of metabolic capabilities of these strains.

3. Probiotics and Prebiotics

3.1 Definition of probiotics

The term probiotic (meaning "for life") is currently used to name bacteria associated with beneficial effects for humans and animals. In 1998, Guarner and Schaafsma proposed a definition of probiotics which was adopted by the Food and Agriculture Organization of the United Nations and the WHO. Probiotic definition currently in use is: "live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the hosts" (FAO/WHO 2002).

This definition has evolved much since the birth of the probiotic concept. Élie Metchnikoff, a Russian biologist, zoologist and protozoologist, was the first to introduce the concept of probiotic microorganisms. In his book entitled "*The Prolongation of Life*", published in 1908, Metchnikoff elucidated the health benefits of lactic acid bacteria (LAB) associated with fermented milk products. Metchnikoff suggested that the intake of LAB contained in fermented milk products, might result in a reduction of toxin-producing bacteria in the gut and that this could increase the longevity of the host. The term probiotic was probably coined for the first time in 1965 by Lilly and Stillwell, to define "substances secreted by one microorganism which stimulates the growth of another", against the concept of antibiotic.

The main probiotic microorganisms belong to the group of LAB including *Lactobacillus* and *Bifidobacterium* (Saulnier et al. 2009; Champagne et al. 2011). However, some no-lactic acid bacteria and yeast have probiotic properties such as *Saccharomyces boulardii*, *Escherichia*, and *Bacillus* (Holzapfel et al. 2001; Burgain et al. 2011).

In order to develop a consensus opinion and better understand the use of the term "probiotic", in 2013 the International Scientific Association for Probiotics and Prebiotics (ISAPP) organized a scientific experts panel. Reviewing the definition of the term probiotic proposed by FAO/WHO in 2002, the panel found that this definition was essentially fine and relevant, however, it clarified the use of the term probiotic in foods and nutrients (Figure 1). In particular, the panel decided to keep live cultures, traditionally associated with fermented foods and for which there is no evidence of health benefits, outside the probiotic framework. Moreover, undefined fecal microbiota transplant were outside the probiotic definition as well. On the contrary, new commensal and consortia comprising defined strains from human samples, with supported evidence of safety and efficacy, are "probiotic".

This Consensus Statement underlined also the necessity to improve the scientific communication to the public and health-care professionals on the benefits of probiotics (Hill et al. 2014).

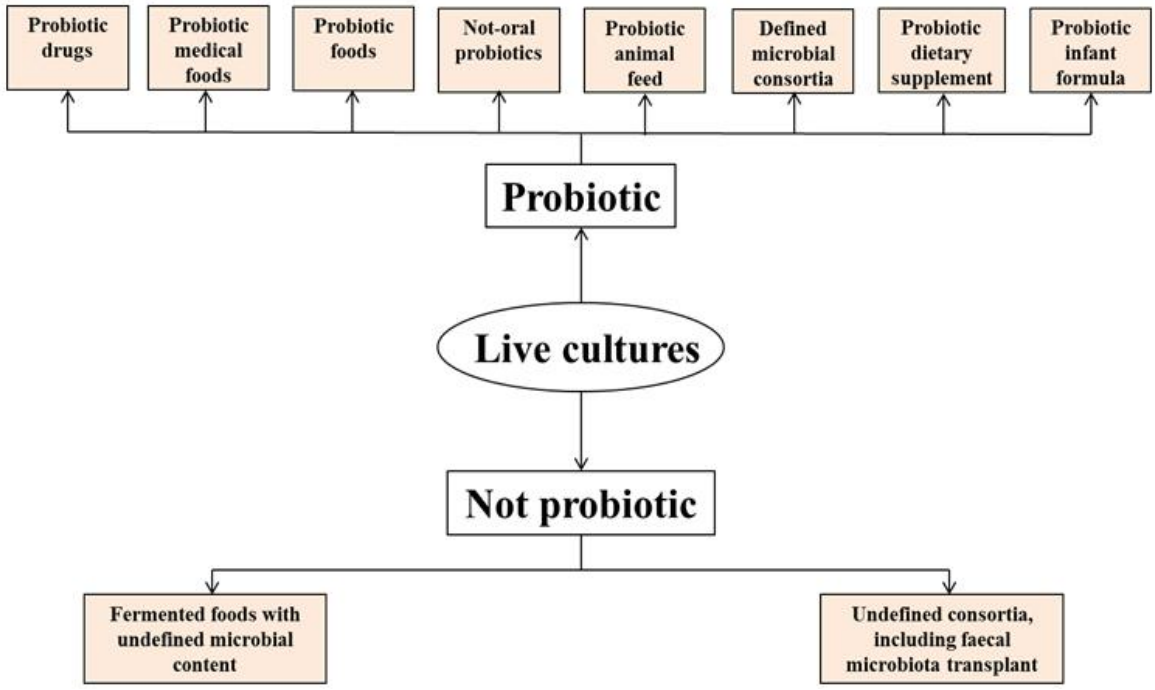


Figure 1 Overall framework for probiotic products (Hill et al. 2014).

3.1.1 Selection criteria of novel probiotic strains

During the past three decades probiotics have been progressing included in various types of food products and also in pharmaceutical preparations for human or animal use (Wallace 2009). However, in order to be tested it is necessary to establish rational criteria for the screening and selection of candidate microorganisms and also to evaluate specific characteristics of efficacy and safety.

Significant progress in legislation evaluating to probiotics has been made in USA, Canada, and Europe (EFSA 2012; HC 2006; FAO/WHO 2002). In USA, safe microorganisms for human consumption must achieve the GRAS status (Generally Recognized As Safe) by the Food and Drug Administration (FDA). In Europe, the European Food Safety Authority (EFSA) has introduced the concept of Qualified Presumption of Safety (QPS) similar in purpose to the GRAS approach.

Several criteria for novel probiotic selection must be taken into consideration (Lee et al. 2008):

1) Strain identification;
2) Safety evaluation;
3) Functional;
4) Technology properties.

Strain identification is an important requirement. It is well established that probiotic properties are strain-related and therefore cannot be extrapolated to other strains belonging to the same species (Soccol et al. 2010). Phenotypic tests represent a first approach to identify potential probiotic bacteria, however, molecular tools are foundamental to establish the strain identity and characteristics. 16S rRNA sequence-based methods and fingerprinting based-techniques could be used in combination. The identified strains, as requested in the European Union, must be deposited in internationally approved culture collections to verify their identity and original properties.

In order to provide a standardized protocol to evaluate the security features, the FEEDAP Panel of EFSA updated peculiar criteria (EFSA, 2012). Antibiotic resistances and transferability of the related genes, in combination with the potential toxic effect of probiotics on the intestinal epithelial cells, are important factors which have to be assessed with respect to healthy claims relating to probiotics.

Several *in vivo* and *in vitro* studies are used to asses these safety characteristics, however, it is important to consider that animal-based studies have major disadvantages. Animal experimentation has a long tradition for risk assessment for new drugs, however, it is difficult to find a suitable animal model to study probiotic strains (Sorokulova et al. 2008). In addition, animal studies do not agree with the bioethical spirit of reducing animal testing in the EU. On the other hand, intestinal cell models, such as human colon tumorigenic cell lines (Caco2, T84 and HT-29), represent an accurate and predictive system close to *in vivo* situation, having a great handling and usability (Cencic and Langerholc 2010).

Among the criteria used to investigate the functional characteristics, adhesion and resistance to stressful GIT conditions are the requisite to allow the persistence and colonization of probiotic strains to the intestinal mucosa. As reported for studies on safety evaluation, cell lines are widely used also for the adhesion assessment (Lee et al. 2008).

Probiotic strains should be able to tolerate stressful conditions of GIT, reaching the colon in a sufficient number to be able to exert beneficial effects (Guarner 2006; Rijkers et al. 2010). Screening of probiotic strains are carried out at different physiological pH and bile concentrations. However, to predict the survival through gastric transit in newborns and young

infants, it is important to consider that the gastric juice is close to neutrality with respect to adulthood (Bergman 2013). Considering the strong effect of gastric juice on probiotic strains, a strategy to improve survival of strains after oral intake upon gastric transit is to make these strains gastro-resistant through a coating material (Del Piano et al. 2011). When in duodenum the coating is disorganized, the survival to bile salts results essential for the generated free cells. Bile is a heterogeneous mixture of organic and inorganic compounds (bile salts, phospholipids, cholesterol, bilirubin and proteins) released by the liver into the duodenum during digestion, facilitating the emulsification and absorption of nutrients from the diet (Begley et al. 2005; Ruiz et al. 2013). A part of bile salts reaches the colon and influence the microbiota with a strong antimicrobial activity as well as DNA damage.

In order to minimize the antimicrobial activity of bile salts, some bacteria have developed many defense strategies. Several *in vitro* studies have shown the ability of strains belonging to bifidobacteria to modulate the expression of membrane proteins, creating a protective layer in response to bile effects (Ruas-Madiedo et al. 2005). In addition, specific bile resistance mechanisms have been described in intestinal bacteria such as bile efflux and bile salt hydrolysis (Begley et al. 2006; Piddock 2006; Gueimonde et al. 2009).

Even though bile tolerance is strain-specific, the potentially probiotic strains could progressively adapt to the presence of bile salts through the over-expression of several genes involved in the bile tolerance (Burns et al. 2010; Ruiz et al. 2013).

A beneficial effects to the host are observed when probiotics are consumed in adequate quantities (a minimum of 10^9 viable cells per day). Thus, the probiotic strains must have good technological properties, so that they can be grown on a large scale and have a longer shelf life (da Cruz et al. 2007; Ouwehand and Vesterlund 2003). In addition, when probiotics are used in food, such as fermented products, they have to withstand stresses such as variations in water activity, pH, oxygen content, and temperature and simultaneously should increase the taste (Forssten et al. 2011).

3.1.2 Probiotic therapy for the treatment and prevention of paediatric diseases

Manipulation of gut microbiota using probiotics in infants has been well documented in clinical trials and has shown promising results in the prevention and treatment of paediatric strictly gut dysfunctions such as, diarrhea, necrotizing enterocolitis (NEC) and infantile colics.

Acute diarrhea represents a major problem in paediatrics, conventional treatment is based on oral rehydration solutions that replace fluids lost (Samadi 1998; Kolotzko and Osterrieder 2009).

However, the best-studied clinical research with the application of probiotic bacteria in children has been the treatment and prevention of this disease (Saavedra 2007). Infectious gastroenteritis is a serious cause of morbidity and, occasionally, mortality , especially in developing countries (Wiegering et al. 2011). Bacteria (i.e., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Clostridium difficile*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli*), parasites (i.e., *Cryptosporidium* spp.), virus (mainly rotavirus) and antibiotic treatments have been identified as trigger causes of diarrhea in infants and young children worldwide (Lowenthal et al. 2006; Amisano et al. 2011).

Oral administration of selected probiotics has shown positive effect in the prevention of diarrhea disease leading to a reduction in frequency of infections. Saavedra et al (1994), in a double-blind, placebo-controlled trial, treated infants who were admitted to a chronic medical care hospital with standard infant formula or the same formula supplemented with a combination of *Bifdobacterium bifidum* and *Streptococcus thermophilus*. It was reported a statistically significant reduction of the incidence of acute diarrhoea in infants feeding with infant formula supplemented. Moreover, in two independent studies, it was found a lower risk of developing antibiotic-associated diarrhea in newborns treated with *B. lactis* associated to *S. thermophilus* (Corrêa et al. 2005) or with *L. rhamnosus* GG (Szajewska et al. 2006), compared to placebo treatment.

Compared to bacterial infection, that due to rotavirus is more severe and often associated with a more complicated progression. Randomized controlled trials in children hospitalized for rotavirus diarrhea and administered with products containing multiple probiotic strains, have established the efficacy of the treatment to decrease the duration and severity of diarrhea and other clinical symptoms such as vomit and fever (Grandy et al. 2010; Vandenplas and De Hert 2011).

Some studies have also been performed to investigate the potentiality of probiotic administration to prevent or treat the necrotizing enterocolitis (NEC). NEC is an inflammatory necrosis of the intestine and represents a major cause of morbidity in preterm infants (Hunter et al. 2008). It is characterized by necrosis and inflammation of intestine whose mucosal barrier results altered. Systemic shock and rapid death can occur in severe cases. Many risk factors are associated with NEC, such as prematurity and bacterial colonization (Claud 2001; Lee and Polin 2003). As it has been already reported in section 1.2, the gut of healthy newborns is colonized by various probiotic strains ascribed to the *Bifidobacterium* and *Lactobacillus* genera. On the contrary, in preterm newborns some pathogenic bacteria, such as enterobacteria and enterococci,

predominate the gut and can be associated with NEC (Mai et al. 2011). Actually, the clinical therapy for NEC is focused on the reduction of symptoms but there are not yet specific strategies of prevention and treatment for this pathology (Schnabl et al. 2008).

Many reports suggested that enteral supplementation with a variety of probiotic organisms on preterm infants can reduce the NEC incidence and NEC-associated mortality (Lin et al. 2008; Caplan 2009). In 2010, an updated systematic review and meta-analysis on 11 randomized controlled trials, confirmed the benefits of probiotic supplements in reducing the incidence of NEC and the associated risk of death in preterm infants (Deshpande et al. 2010).

In 2005, a clinical trial was conducted to evaluate the prophylactic effect on the development of NEC of a mixture of probiotic strains, *B. longum* subsp. *infantis*, *B.bifidum* and *S. thermophiles*, in infants weighing less to 1.5 Kg. The study found that probiotic supplementation reduced both the incidence and severity of NEC (Bin-Nun et al. 2005). Moreover, similar results were obtained in the study of Braga et al. (2011) who administered two probiotics strains, *B. breve* and *L. casei*, in low-birth-weight preterm infants.

Infant colic is characterized by inconsolable crying, frequently accompanied by flushing of the face and meteorism (Cirgin Ellett 2003). Although is a common gastrointestinal dysfunction of infants, about 10-30% of infants are affected, infant colic pathogenesis is not well understood. However, an aberrant intestinal microbiota has been proposed as a major determinant for the pathogenesis. During the first months of life, the colicky infants have a lower level of bifidobacteria and lactobacilli and higher counts of Gram-negative bacteria (e.i., *Escherichia*, *Klebsiella*, *Serratia*, *Vibrio*, *Yersinia*, *Pseudomonas* and *Enterobacter*) compared to healthy infants (Savino et al. 2004; de Weerth et al. 2013). Among these pathogenic bacteria, some species are able to produce gas through acid fermentation and thus promote gassy colic (Savino et al. 2009).

With respect to the application of probiotic to colic treatment, (Savino and Tarasco 2010), administering to breastfed colicky infants a strain of *L. reuteri*, demonstrated a positive modulation of the gut microbiota and a reduction of gas colic symptoms. In contrast to these findings, a recent clinical trial, investigating the same *Lactobacillus* strain which was tested previously by Savino et al (2010), did not find the same positive effects (Sung et al. 2014). Moreover, the application of bifidobacteria against infant colic symptoms is not reported *in vivo* and restricted only in a study, *in vitro*, showing a great inhibitory ability of selected strains, against the growth of some gas-forming coliforms (Aloisio et al. 2012). In this contest, additional clinical studies should allow to validate the efficacy of probiotic strains against colic.

To conclude, probiotics are increasingly being used for their health benefits, however, not all the probiotic strains drive a targeted therapeutic effect, thus, it is important to selection different strains which can have very specific properties. Therefore, clinical trial results from one probiotic strain in one population cannot be automatically generalized to other strains or to different populations.

Moreover, the probiotic effects may depend from the health status of the host, dosage and duration of administration. Furthermore, several studies have showed the synergic outcome of probiotic mixtures consisting also of strains belonging to different genera and species.

3.2 Definition of prebiotics

Gibson and Roberfroid have introduced since 1995 the concept of prebiotic, which was defined as "specific food components not digested and not absorbed in the small intestine but fermented in the large intestine by microbiota". This definition has been modified by (Saad et al. 2013) who presented the concept of prebiotic as non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health. The prebiotics must withstand to mammalian hydrolytic enzymes and gastric acidity and reach the colon, where they are fermented by gut microbiota. The microbial fermentation brings to modulate the activity and stimulate the intestinal bacterial growth, in addition it should induce beneficial luminal/systemic effects within the host (Manning and Gibson 2004; Laparra and Sanz 2010; (Roberfroid et al. 2010). The main goal of prebiotics is the increase in number and activity of beneficial bacteria such as bifidobacteria and lactobacilli, and a consequent reduction of putrefactive microorganisms or potentially pathogenic bacteria such as clostridia and enterobacteriaceae (Campbell et al. 1997; Rycroft et al. 2001; Saulnier et al. 2009).

3.2.1 Chemical description and intestinal health benefits

Most identified prebiotics, normally occurring in human and animal diet, are carbohydrates, and the most promising are non-digestible oligosaccharides (NDOs) with a low degree of polymerization (DP) (Yun 1996). They have been defined as consisting of 2 up to 30 monosaccharide units whose anomeric C atom (C1 or C2) has a configuration that makes their glycosidic bounds non-digestible to the hydrolytic activity of the human digestive enzymes (Delattre and Vijayalakshmi 2009; Roberfroid and Slavin 2010).

DP and type of glycosidic bonds between the monosaccharide units, which are fructose, galactose, glucose and/or xylose are responsible for the specific metabolic use of NDOs by improving and modulating the growth of intestinal bacteria (Rycroft et al. 2001; Roberfroid 2007).

Many prebiotic fibers are obtained from raw vegetable materials, while others are produced by chemical or enzymatic processes (Delattre and Vijayalakshmi 2009). Among NDOs, the most important fibers are fructo-oligosaccharides (FOS, oligofructose, inulin), galacto-oligosaccharides (GOS) or trans-galactooligosaccharides (TOS), lactulose, gluco-oligosaccharides, glycol-oligosaccharides, lactitol, isomalto-oligosaccharides, malto-oligosaccharides, xylo-oligosaccharides, stachyose, raffinose, and sucrose oligosaccharides (Patterson and Burkholder 2003). Additionally, several polysaccharides such as Arabinogalactans (AG) and partially hydrolysed guar gums (PHGG), have a great capability to stimulate positively the growth of colonic LAB and bifidobacteria.

3.2.1.1 Fructo-oligosaccharides (FOS) and inulin

FOS and inulin are chains of fructosyl units linked to a terminal α-D-glucose residue (GFn). The fructosyl units are linked by bonds β(1→2); the glucose is linked by bonds β(1→1). The only chemical difference between FOS and inulin is the different degree of polymerization (DP). Whereas the chain lengths of FOS range from 3 to 10 units, the inulin has a DP which can reach up to 60 units (Figure 2).

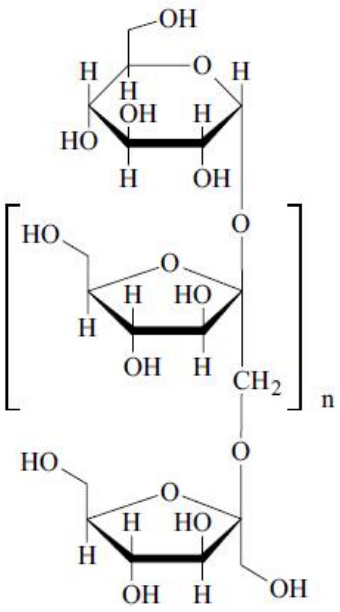


Figure 2 General structure of FOS (n = 0 - 7) and inulin (n = 0 – 57).

The characteristic bond β(1→2) in the fructosyl units, prevents FOS and inulin from being digested with respect to common carbohydrate conferring a high nutritional value.

FOS and inulin can be extracted from several vegetables such as, chicory, artichoke, onion, garlic and Jerusalem artichokes.

Furthermore, FOS can be manufactured: i) by sucrose (GF), using the transfructosylation activity of the enzyme β-fructofuranosidase (or fructosyltransferase) which produces increasing length oligomers such as, 1-kestose (GF2), 1-nystose (GF3) and 1F-fructosylnystose (GF4); ii) by controlled enzymatic hydrolysis of the extracted polysaccharide inulin.

Several studies, *in vitro* and *in vivo*, have widely established the prebiotic properties of FOS and inulin which to date are the most commercialized and used.

Intake of FOS leads to an increase of the count of bifidobacteria, *in vivo*, and reduces that of *Bacteroides* spp. (Kolida et al. 2002). In addition, FOS and inulin have other beneficial effects in the host, such as modulation of mineral metabolism, modulation of immune system and decrease of cholesterol levels.

Studies in humans have shown the possible effect of these carbohydrates to enhance calcium absorption and bone mineral density (Macfarlane et al. 2006). The immune-modulation in the intestine has been also pointed out by several studies. After administration of inulin and FOS, various parameters of the immune system are altered, such as secretion of IL-10 and interferon (IFN)-γ by Peyer's patch (Hosono et al. 2003), NK cell activity, lymphocyte proliferation, immunoregulation of intestinal IgA, a general development of GALT (Pierre et al. 1997).

A series of animal studies demonstrate that inulin affect the metabolism of the lipids decreasing triglyceridaemia and cholesterolaemia. Studies on humans largely confirm the animal experiment results demonstrating mainly a reduction in triglyceridaemia and only a relatively slight decrease in cholesterolemia (Delzenne et al. 2011).

3.2.1.2 Galacto-oligosaccharides (GOS)

GOS are made from of 2 to 6 sugar oligosaccharides formed by transgalactosylase activity of β-galactosidase on lactose (disaccharide composed of galactose and glucose bonded by a β -1,4-bond); these are frequently referred to as transgalactosylated oligosaccharides (TOS).

The enzyme β-galactosidase works by transferring galactose from lactose to water. Under condition of high lactose concentration, the enzyme uses lactose as an alternative acceptor to water resulting in the formation of galactooligosaccharides (Rastall et al. 2002). The lactose used as substrate for GOS production is usually purified from cow's milk.

A major component of the GOS is 4'-galactosyl lactose (4'-GL) having a structure composed of lactose and galactose bonded through a β -1,4-bond. The general structure is: Gal β 1-4(Gal)n β 1-4Glu (Figure 3).

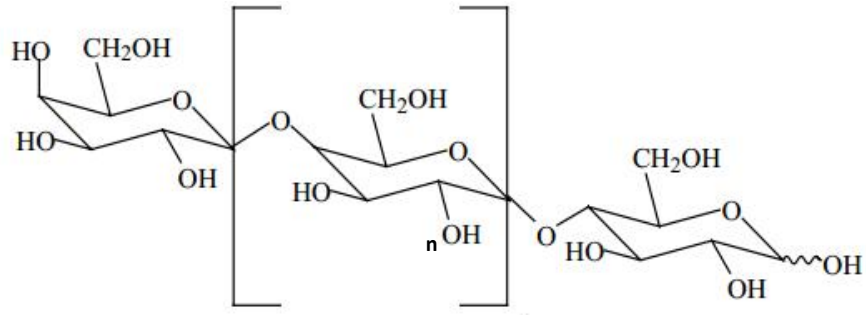


Figure 3 General structure of 4'-galactosyl lactose (n = 1 - 3)

GOS resist to the hydrolysis of intestinal enzymes and thus they reach the colon undigested. Several studies in humans, both in adults and in infants, have shown the selective stimulation of GOS in the growth of the health-promoting bacteria such as bifidobacteria and lactobacilli. Ben et al. (2008) evaluated the effects of an infant formula enriched with GOS (0.24 g/100 mL) on the intestinal microbiota in 371 healthy and in term infants. After 3 month of GOS-formula feeding, *Bifidobacterium* spp. and *Lactobacillus* spp., acetic acid and stool frequency were significantly increased, on the contrary fecal pH was decreased compared to the infants feeding with the formula without GOS. Additionally, compared to human milk, the GOS-formula did not show differences, showing, thus, its effectiveness when used in infants. To date, fermented milk products containing probiotic bacteria with added GOS are commercially available in Japan and in Europe, thus, infant nutrition is a promising field of application of GOS.

GOS have demonstrated positive effects on calcium absorption and have prevented bone loss in some animal research or in human studies (Chonan et al. 2001; Whisner et al. 2013).

Relief of constipation and support to natural defences in human have also been pointed out (Rycroft 2002; Boehm 2008).

3.2.1.3 Arabinogalactans (AG) and partially hydrolyzed guar gum (PHGG)

The polysaccharides AG and PHGG belong to the hemicellulose fibers and have a low molecular weight and an irregular and branched structure.

AGs are water-soluble polysaccharides found in plants, fungi and bacteria and the dietary intake of this compound comes from carrots, radishes, tomatoes, pears and wheat. AG, derived from the larch tree, is commercially available as fiber ingredient and it is considered as non-digestible

soluble dietary fiber. Chemically, it is composed of the sugar galactose (β-1-3 with branches β-1-6) and arabinose (β-1-3).

PHGG is a soluble fiber produced from the seed of guar bean that completely dissolves in water and is fermented in the colon liberating SCFAs. Chemically, guar gum (or galacto-mannan) is composed of galactose (α-1-6) and mannose (β-1-4).

AG fibers possess interesting prebiotic properties, being fermented by gut microbiota and resulting in the production of short-chain fatty acids, primarily butyrate and propionate. Moreover, in subjects administered with AG a significant increase of *Lactobacillus* spp., compared to the control group and a well tolerability were observed (Robinson et al. 2001).

The ability of AG to enhance immune system performance has been evidenced. Larch arabinogalactan obtained from *Larix occidentalis* enhanced NK cell cytotoxicity and phagocytic capacities of macrophages and lymphocytes (Hauer and Anderer 1993).

PHGG supplementation in infants leads to decrease some functional bowel disorders, such as irritable bowel syndrome (IBS) and abdominal pain symptoms (Parisi et al. 2005; Romano et al. 2013), in addition, have reported a rise in the proportions of bifidobacteria and lactobacilli after PHGG supplementation in human (Tuohy et al. 2007).

3.2.2 Human milk oligosaccharides (HMO)

The oligosaccharides of human milk (HMO), one of the major components of breast milk, are partially digested in the small intestine and therefore they reach the colon, where they exert a prebiotic effect. They are able to stimulate selectively the growth of bifidobacteria contributing to the formation of the characteristic composition of the intestinal microbiota in breast-fed infants (Coppa and Gabrielli 2008).

During the first two weeks after delivery, the human's milk has a content in oligosaccharides of about 20 g/L and when it becomes mature and reaches stability achieves a concentration ranging from 12 to 14 g/L, on the contrary, cow's milk, which is commonly used in the preparation of infant milk formulas, has a low content of oligosaccharides, about 1 g/L (Coppa et al. 2006).

HMOs are structurally very complex and have a huge diversity, almost 200 oligosaccharides have been identified, of which over 80 are now fully characterized from the structural point of view (Tao et al. 2011; Yang et al. 2011). HMOs are carbohydrates composed of glucose and galactose with added several units of N-acetyl-glucosamine further decorated by fucose. This structure of monosaccharides can bound to one or more molecules of sialic acid.

The presence of acid sialic and fucose in terminal positions confers HMO as non-digestible oligosaccharides by digestive intestinal enzymes. This oligosaccharides, thus, reach the colon

intact where they are metabolized by the intestinal bacteria which possess proper enzymes (Marcobal et al. 2010).

The bifidogenic effect of HMO is restricted to some strains which have preference in oligosaccharide consumption, among them *B. infantis* and *B. bifidum* possess specific enzymes, such as fucosidase and sialidase, to deconstruct the HMO polymer. Genomic analysis of these strains have shown the induction of specific gene clusters associate with HMG stimulation and consumption (Sela and Mills 2010; Turroni et al. 2012b). HMO have shown to modulate the intestinal immune cells by a direct stimulation of their sugar receptors (Eiwegger et al. 2010).

In addition to HMO, human milk has a wide range of immune-modulatory factors including Igs, lysozyme, lactoferrin, cytokines and lymphocytes (Penttila 2010; Walker 2010).

Moreover the prebiotic effect of human milk is also enhanced by components other than oligosaccharides, such as lactoferrin, nucleotides, lactose (Coppa and Gabrielli, 2008).

Objectives

My Ph.D position was supported by the Emilia-Romagna Region within the program Global Grant Spinner 2013. The work described in this dissertation is part of a larger project entitled "Functional foods and nutraceuticals applied to human and animal health: probiotics, prebiotics and activation of phytochemicals". The goal of Global Grant Spinner 2013 is to support the qualification of human resources in the field of research and technological innovation and to create an inter-university networks of Ph.D projects.

My activity has investigated the early colonization of the gut with a particular focus on bifidobacteria. Gut microbial acquisition during the early stage of life is an extremely important event. Since birth, the microbiota evolves into a complex ecosystem which will affect the health status of the host. In this context, the healthy properties of the genus *Bifidobacterium* have a central function in newborns. A preliminary work performed in our laboratory has led to the isolation and characterization of four *Bifidobacterium* strains (three *B. breve* and one *B. longum*) which are potential candidates to be used as probiotics in infants (Aloisio et al., 2012). My Ph.D research was a follow up of this work.

The aim of the work proposed in this thesis was therefore to explore the dynamics of the gut microbial colonization in the early stage of life and to suggest possible strategies to maintain or restore a correct balance of gut bacterial population in infants.

To date, the application of probiotics and prebiotics for the prevention or treatment of infant disease is an emerging area of the applied microbiology, furthermore, it has a growing interest for the food and pharmaceutical industry. The first step of this work was to review the latest advanced studies on the use of probiotics and prebiotics in infants; particular emphasis was given to bifidobacteria (**Paper 1** and **2**).

Among the intestinal disorders that affects newborns, gas colics are quite diffused. Although the pathogenesis of this disease is not well understood, it has been evidenced, using both culture-dependant and molecular investigations, higher counts of Gram-negative bacteria, in particular gas-producing coliforms, and less proportions of LAB and bifidobacteria in colicky infants compared to healthy infants. **Paper 3** evaluated the capability of a *Bifidobacterium breve* strain, previously characterized (Aloisio et al., 2012), to reduce the amount of *Enterobacteriaceae* in fecal cultures derived from a colicky infant, using a continuous culture fermentation approach.

The stimulation of the growth of beneficial bacteria may be a suitable strategy to prevent some pediatric disease and consequently to maintain a correct bacterial balance. For this reason, in order to develop a synbiotic product for infants, the ability of ten commercial prebiotic fibers (two galactooligosaccharides, one fructooligosaccharide, four inulins, one glucooligosaccharide, one arabinogalactan and one partially hydrolysed guar gum) to stimulate selectively the growth of four bifidobacteria strains (three *B. breve* and one *B. longum*) with respect to potentially harmful bacteria of the gut, was evaluated. In addition, the capacity of the four bifidobacteria strains, previously characterized (Aloisio et al., 2012) to survive in simulated gastro-intestinal conditions was evaluated in order to check the most suitable way of administration to infants (**Paper 4**).

A large part of my Ph.D research activity has been dedicated to the understanding of how intrapartum antibiotic prophylaxis (IAP) can affect gut colonization in infants. Positive pregnant women to Group B *Streptococcus* (GBS), during delivery, can transmit this bacteria to newborns. The infection is one of the major causes of neonatal morbidity and mortality, therefore, in order to reduce its incidence, IAP with ampicillin is routinely carried out in GBS-positive women. However, the impact of IAP on the early microbial colonization in infants has not been clarified yet. In order to evaluate the microbiota modulation in infants due to IAP treatment, several experimental approaches were applied, which include the counts of several microbial groups in infants using quantitative PCR, the analysis of bifidobacteria biodiversity with Denaturing Gradient Gel Electrophoresis and a next generation sequencing approach (**Paper 5**, **6** and **7**). Additionally, the **paper 5** investigated the antimicrobial activity of the same four *Bifidobacterium* spp. strains, previously considered in the paper 4, against four strains of *Streptococcus agalactiae* (isolated from vaginal swabs of GBS-positive women). The specific aim of this study was to evaluate, in GBS-positive women, alternative actions of prevention or treatment involving probiotic application.

PAPER 1

Chapter 26: Infant Development, Currently the Main Applications of Probiotics And Prebiotics?

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Abstract:

Research on probiotics and prebiotics for use in infants is very active and results on their efficacy to prevent and combat several diseases are at present available. Bifidobacteria and lactobacilli are considered beneficial bacteria for the gut, the former being the predominant group of healthy breast-fed newborns. One of the major area of probiotic research in children has been the treatment and prevention of diarrhea. Moreover, a large number of infant pathologies, both enteric (infantile colics, necrotizing enterocolitis, celiac disease) and not strictly enteric (allergies, obesity, neurologic disease) have revealed promising preventive and therapeutic effects of probiotics, although these applications need additional experimental evidences. Recent studies have shown that probiotic strain characteristics are crucial to reach a targeted therapeutic effect. One of the major aspect affecting the gut microbial composition of breast-fed neonates is the presence of oligosaccharides in breast milk. These molecules exert a prebiotic effect which is crucial for the development of a healthy gut microbiota. Research studies have been focused on the selection of fibers possessing a prebiotic role similar to human milk oligosaccharides. Galactooligosaccharides and fructoligosaccharides are abundantly used in infant formula, frequently as mixtures of the two molecules. Several studies have shown that the capability of stimulating beneficial bacteria and of shaping the gut microbiota is similar to that of breast milk. On the contrary, studies regarding the use of prebiotics in infants for the prevention of allergies showed contradictory results. Therefore, it is possible to conclude that children are a very important target, if not the main one, for probiotic and prebiotic administration and the European industry is aware of that.

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26.1 Introduction

Probiotics and prebiotics constitute a central growing market for the food/pharmaceutical industry and for the baby food industry as well. The industrial significance of these health promoting bacteria and molecules has driven a lot of research studies aimed at understanding their functionality and activity. The children associated market is of great relevance, because infants are very susceptible to diseases and non chemoterapeutic treatments are particularly looked forward for them.

Probiotic and prebiotic research is moving forward on two fronts: basic science, i.e. laboratory studies planned to elucidate the mechanisms through which these supplements exert their activity, and clinical trials to evaluate the safety and efficacy of probiotics in various medical conditions. Many early clinical trials of probiotics/prebiotics had methodological limitations, and definitive clinical evidence to support using specific probiotic strains for specific health purposes is sometimes lacking. Nevertheless, there is preliminary evidence for several uses of probiotics and prebiotics, and more studies are under way. This chapter is focused on the use of probiotics and prebiotics in infants for the treatment of infant diseases and, in the case of prebiotics, will describe attempts aimed at making formula milks as close as possible to human milk to overcome limitations connected to the lack of feeding with maternal milk. The basis of probiotics/prebiotics use is that the health status of the gut in infants is extremely important for the well-being of the whole organism in the successive stages of life. The definition of an equilibrated gut microbiota composition in the early stages of life and, therefore, the understanding of how colonization occurs in newborns, are of crucial importance.

26.2 Gut microbiota acquisition in infants

Three different successive phases in the composition of the intestinal microbiota can be described almost in all individuals: i) the birth and the whole period of liquid diet; ii)the weaning time up to adulthood, when the introduction of a solid diet determines profound changes giving rise to a stable community; iii) the elderly period, when further strong changes in the microbiota occur deriving from both physiological modification of the gastrointestinal tract and an unbalanced diet (Claesson et al. 2011) Studies on gnotobiotic mice have illustrated the essential role of the gastrointestinal microbiota in normal gut development and it is argued that the microbial diversity of the human gut is the result of co-evolution between microbial communities and their hosts (Ley et al., 2006). It is well assessed that the intestinal microbiota of the newborn is a complex ecosystem composed of numerous genera, species and strains of bacteria, performing various unique activities affecting colonic and systemic physiology and that its

establishment begins soon after birth. Birth brings about an immediate end to the sterility of the fetus environment and, within a few hours, bacteria start to appear in the feces. The first microbial population the newborn comes into contact with are the maternal intestinal and vaginal microbiota; successively, the newborn is exposed to microbes from the environment. Still, the first microbial colonization of the infant gastrointestinal tract is a remarkable episode in the human lifecycle and it is well known that several factors, in addition to the maternal microbiota, influence the gut colonization by microorganisms, including the mode of delivery, the type of infant feeding, the perinatal newborn circumstances (hospitalization and chemotherapy treatments) (Biasucci et al., 2010; Di Gioia et al. 2014).

The first bacteria healthy newborns come in contact with are facultative anaerobes (*Staphylococcus* spp., *Enterobacteriaceae* and *Streptococcus* spp.), because of the positive oxidation/reduction potential of the neonates' intestine at birth. These bacteria consume oxygen paving the way to strict anaerobes such as members of the *Bifidobacterium*, *Bacteroides* and *Clostridium* genera (Biasucci et al., 2010). Epidemiologic studies performed in the last decade and focused on the determination of factors affecting gut composition (e.g. the KOALA study, Penders et al. 2006) have shown that that anaerobic colonization may be delayed in caesarean section delivered infants. Additionally, an increased incidence of *Clostridium perfringens* and *Clostridium difficile* in caesarean section newborns is reported in relation to the hospital environment. Another factor influencing composition of the intestinal microbiota in neonates is the type of feeding (Penders et al. 2006). In full term breastfed neonates *Bacteroides* spp. and bifidobacteria can appear 4 days after birth and after 1 week they their counts increase rapidly to constitute 80%-90% of the total bacteria, whereas the microbiota of formula-fed infants is more complex, with *Bifidobacterium* spp., enterobacteria and *Streptococcus* spp. in similar proportion. Another notable difference is that formula fed infants have much higher counts of *Clostridium* spp than breast fed infants (Penders et al., 2006). An important difference is the relative buffering capacity of the two feeds. Breast milk has poor buffering capacity, compared with formula milk, and this leads to market differences in the colon pH of breast and formula fed infants: 5.1 and 6.5, respectively. This low pH promotes the growth of bifidobacteria and lattobacilli, but is inhibitory to many other bacteria (Tham et al., 2011). Moreover, human milk oligosaccharides, as described in section 24.6.1 of this chapter, are prebiotic agents that selectively encourage the growth of beneficial (probiotic) organisms (Coppa and Gabrielli, 2008). Both adults and neonates are regularly exposed to microorganisms via the diet, but with different effects. The microorganisms entering newborns through breast milk are more likely to colonize than those entering healthy adults possessing a stable climax community. Information

about the isolation and identification of commensal or potential probiotics bacteria, including bifidobacteria, from milk of healthy women are not conclusive. Even though authors are aware that human milk is difficult to sample and microbial contamination can never be totally discarded, some studies have demonstrated the presence of live bifidobacteria in human milk (Martin et al., 2003, Solis et al., 2010).

In contrast with full term neonates, little information concerning the composition of the microbiota in premature infants is available. The inter-individual variability in these subjects is very high and many parameters, such as antibiotic use and diet, may tend to increase study discrepancy. In particular, preterm newborns often need parental feeding, due to the immaturity of their intestine, and they often need respiratory support, they are vulnerable for infections and often require antibiotic treatment. One of the most significant differences between preterm and full term infants microbiota is the colonization of bifidobacteria that are not frequently identified in the first month of life of premature newborns (Westerbeek et al., 2006). This alteration has been linked to the increased risk of severe gastrointestinal disorders such as necrotizing enterocolitis which affects predominantly premature and low weight newborns (Lin et al., 2008).

The gut microbiota of infants, due to change of diet, results more stable and homogenous after weaning and it gradually gets closer to the typical adult microbiota (Magne et al. 2006; Koenig et al. 2011). A large-scale longitudinal study on development and change in the composition of gut microbiota during the process of weaning was carried out by Fallani et al. (2011). Within this study faecal samples of 605 infants (from five European countries), approximately 4 and 6 weeks after the introduction of first solid foods, were collected in order to investigate the association with determining factors such as mode of delivery, previous feeding practices, age of weaning and the impact of possible antibiotic treatment. After 1 month of weaning, bifidobacteria and *Bacteroides* continue to represent the predominant groups. However, the number of detectable *Bifidobacterium* species decreased after weaning together with counts of *C. perfringens* and *C. difficile*, while other strictly anaerobic clostridia increased.

26.3 Association between gut microbiota composition and health status

Because of immature intestinal immune function, the newborn is susceptible to intestinal and systemic infections. An immature intestinal epithelial barrier may predispose infants and children to intestinal inflammatory diseases, such as infectious enteritis, inflammatory bowel disease, and necrotizing enterocolitis. Therefore the development of an healthy microbiota during the postnatal period is critical for the establishment of normal physiology of the intestinal tract. Moreover, a better understanding of the factors that regulate gut barrier maturation may

yield insight into strategies to prevent these intestinal diseases. The microbiota is in close contact with the intestinal mucosa and epithelial surface which is, after the respiratory area, the largest surface of the body, occupying approximately 250-400 m^2 (Nataro 2005). Some anatomical and physiological aspects of the host organism are directly linked to the presence and activity of the resident microorganisms, such as formation of the intestinal walls, production of organic acids and vitamins, stimulation of immune system etc. The presence of abundant bifidobacteria and lactobacilli may provide some protection against incoming of enteric pathogens. The benefits exerted by the strains belonging to *Bifidobacterium* and *Lactobacillus* genera have been widely studied in the last ten years (Laux et al. 2005, Jankowska et al. 2008, Cencic and Langerholc 2010, Montier et al. 2012). They are able to compete for nutrient with enteric pathogens, to strongly adhere to the intestinal mucosa and to stimulate the development of both humoral and cellular mucosal immune system. These and many other features make them excellent probiotics for pediatric use.

26.4 Beneficial bacteria in the newborn gut

26.4.1 Bifidobacteria

The intestinal microbiota of breast-fed newborns is predominantly composed of bifidobacteria. This achievement was already reached in several studies performed in the eighties (Biavati et al. 1984) with the use of traditional plate isolation technique. The most represented species in both breast-fed and formula-fed infants were found to be *Bifidobacterium infantis* (at present classified as *B. longum* subsp. *infantis*), *Bifidobacterium breve*, *Bifidobacterium longum* (at present classified as *B. longum* subsp. *longum*) and *Bifidobacterium bifidum*. *Bifidobacterium pseudocatenulatum* and *Bifidobacterium catenulatum* were also present although in a lower number in both type of newborns, whereas *Bifidobacterium dentium* was evidenced only in breast-fed neonates. In the last twenty years the development of molecular techniques has completely revolutionized the way of performing microbial ecology studies. Thermal gradient gel electrophoresis (TGGE) studies of genomic DNA amplified from infant feces collected during the breast feeding period confirmed that bifidobacteria were the predominant group, precisely *B. infantis*, *B. longum* and *B. breve*, whereas in the post-weaning period a decrease in the *Bifidobacterium* population was observed (Magne et al. 2006). Real time PCR analyses of targeted *Bifidobacterium* species indicated that the number of species in breast-fed newborn was initially higher. *Bifidobacterium animalis* and *B. dentium* were not detectable in samples irrespective of the type of feeding , whereas *B. longum* subsp. *infantis*, *B. breve, B. bifidum* and

B. longum subsp. *longum* were detected in all samples, *B. longum* subsp. *infantis* being the major species found. *Bifidobacterium adolescentis,* which is most commonly found in adults, was present only in formula-fed infants (Klaassens et al. 2009). Further insights in the infant gut microbiota were recently carried out using next generation sequencing targeted on two hypervariable regions of the 16S rRNA gene by Turroni et al. (2012). This study elucidated that the most abundant class in infant faecal samples was *Bifidobacteriales*, being present at 80.6%. The predominant bifidobacterial species detected were *B. longum* and *B. bifidum* at 56.2% and 10.7%, respectively. Regarding the inter-individual variability in the infant gut microbiota, the statistical analyses performed revealed a large conservation of members of the *Actinobacteria* with a high proportion (ranging from 21.7% to 90.6%) belonging to the *Bifidobacteriaceae* family. In particular, the *B. breve* species was always detected with an average of 5.5% of total reads. In contrast, *B. adolescentis* was detected in a relatively high average percentage (3.4%), but it was only present in about 2% of the subjects.

26.4.2 Lactobacilli

Together with *Bifidobacterium* and *Bacteroides*, the *Lactobacillus* genus is one of the major component of the breast-fed newborn intestinal microbiota. However, the distribution of the different *Lactobacillus* species in infant has not been studied in details as in the case of bifidobacteria. Satokari et al. (2002) carried out the analysis of *Lactobacillus* diversity in breast-fed and bottle-fed newborns using denaturing gradient gel electrophoresis (DGGE) approach. The analysis revealed that the predominant *Lactobacillus* population consisted of one or two dominant species and *L. acidophilus* resulted to be the most common species irrespective of the type of feeding. A further study has been focused on the distribution of *Lactobacillus* species in faeces of breast-fed newborns and newborns receiving standard formula milk or standard formula milk supplemented with galactooligosaccharides and fructooligosaccharides (Haarman and Knol, 2006) using real time PCR with primers and probe sets designed on the intergenic spacer of 16S-23S rRNA gene. This approach allowed to have a more detailed analysis of *Lactobacillus* species because sequences are less conserved than 16S rRNA gene sequence. The *Lactobacillus* species distribution in breast-fed was mainly composed of *L. acidophilus, Lactobacillus casei and Lactobacillus paracasei*. The same distribution was also found in newborns receiving prebiotic supplementation. On the other hand supplementation with standard formula resulted in a different *Lactobacillus* distribution with more *Lactobacillus delbruekii* and *Lactobacillus reuteri* and less *L. paracasei* and *L. acidophilus. Lactobacillus fermentum, Lactobacillus plantarum*, and *Lactobacillus rhamnosus* were also present at a very low

percentage at the beginning of the intervention period and these species seemed to disappear completely during the intervention in all feeding groups. Changes in the distribution of *Lactobacillus* species has been also demonstrated by Salminen et al. (2014). The authors carried out a double-blind , randomized prospective study of 21 to 30–day old healthy and vaginally born infants showing that *L. paracasei* and *L. rhamnosus* increased in newborn fed with formula milk supplemented with galactooligosaccharides.

26.5 Probiotics and their pediatric use

One of the primary areas of probiotic research in children has been the treatment and prevention of bacterial acute diarrhea and antibiotic associated diarrhea (Wiegering et al. 2011). Moreover, a large number of infant pathologies, both enteric and not strictly enteric, have revealed promising preventive and therapeutic effects of probiotics, although these applications need additional experimental evidence (Chen and Walker, 2011; Taibi and Comelli, 2014). Therefore, research is currently going on for the use of probiotics in necrotizing enterocolitis, infantile colics and celiac disease, as described below and briefly summarized in Table 1.

Table 1 Overview of probiotics applications in the prevention and treatment of gastrointestinal diseases

Pathology	Probiotic microorganism	Reported effect(s)	References
Acute diarrhea	*Lactobacillus rhamnosus* GG	Reduced risk of diarrhea	Szajewska et al. 2006
	B. animalis subsp. *lactis*, *L. acidophilus,* *L. rhamnosus* strains	Decreased duration of diarrhea	Grandy et al. 2010
	B. longum susp. *infantis*	Increased inhibition of rotavirus virulence (*in vitro* study)	Munoz et al. 2011
	B. animalis subsp. *lactis* plus *B.longum* subsp. *Infantis*, *Streptoccoccus thermophilus*,*Lactobacillus rhamnosus, Lactobacillus acidophilus* strains	Decreased duration of diarrhea	Vandelplas and Hert 2011
	L. reuteri DSM 17938	Reduced incidence of nosocomial diarrhea	Wanke and Szajewska 2012
	B. breve DSMZ24706, *B. breve* DSMZ24707, *B. breve* DSMZ24708, *B. longum* DSMZ24709	Increased inhibition of diarrhea pathogens (*in vitro* study)	Aloisio et al. 2012

	B. longum subsp. *infantis* 35624	Reduced *Salmonella* spp. infection (*in vitro* study)	Sydmonds et al. 2012
	L. ruminis SPM0211	Decreased duration of viral diarrhea	Kang et al. 2013
Infantile colics	*B. breve* DSMZ24706, *B. breve* DSMZ24707, *B. breve* DSMZ24708, *B. longum* DSMZ24709	Antimicrobial activity against gas forming coliforms (*in vitro* study); clinical trial is on going	Aloisio et al. 2012
	L. reuteri DSM 17938	Reduced gas colics symptoms	Savino et al. 2010
	L. reuteri DSM 17938	Reduced time of crying	Szajewska et al. 2013
	L. reuteri DSM 17938	No effect on treatment of infant colics	Sung et al. 2014
Necrotizing enterocolitis	*B.bifidum* strain in Infloran product	Reduced incidence of NEC and death	Lin et al. 2008
	B.breve strain (Yakult preparation)	Increased intestinal motility	Braga et al. 2011
	L. reuteri strain	Reduced frequency of sepsis	Oncel et al.2013
Celiac disease	VLS#3 product *(B.longum susp. longum, B. breve, B.longum* subsp. *infantis, S. thermophilus*, *L. plantarum*, *L. casei L.acidophilus*, *L. delbrueckii*)	Decreased toxicity of gluten (murine model and human cell lines)	De Angelis et al. 2006
	VLS#3 product	Decreased toxicity of gluten during food processing	Kaur et al. 2002
	B. animalis subsp *lactis and L. lactis*	Reduced damage induced by gliadin (human cell lines)	Linfors et al. 2008

It is likely that, in the future, probiotics will become a consolidated therapy for these diseases. In addition, a number of pathologies, which are not strictly gut dysfunctions, have in recent years been correlated to alterations of the gut microbiota, such as allergy, obesity, neurological and psychiatric diseases (Table 2).

Table 2 Overview of potential probiotics applications in infant diseases not directly related to the gastrointestinal tract

Pathology	Probiotic microorganism	Reported effect(s)	References
Allergies	*B. breve* BB99, LGG, *L. rhamnosus* LC705	Reduced eczema caused by cow's milk allegy	Viljanen et al. 2005
	B.longum BL999, *L. rhamnosus* LPR	No effect on prevention of eczema	Soh et al. 2009
	B. breve BB99, LGG, *L. rhamnosus* LC705	Decreased incidence of IgE-associated allergy	Kuitunen et al. 2009
	L. johnsonii EM1	Reduced perennial perennial allergic rhinitis	Lue at al.2012
Obesity	*Bifidobacterium* spp. strains	Increased glucose-tolerance and decreased proinflammatory cytokines (murine model)	Cani et al. 2007
	VLS#3 product	Increased hepatic natural killer T-cell (murine model)	Ma et al. 2008
	Bifidobacterium spp. strains	Reduced serum and liver triglyceride (murine model)	Yin et al. 2010
	Mixture of three *Bifidobacterium* strains	Reduced serum cholesterol level (murine model)	An et al. 2011
Neurology and psichiatry	*B. infantis* 35624	Reduced hyperactivity (murine model)	Sudo et al. 2004
	B. infantis 35624	Increased level of plasma tryptophan (murine model)	Desbonnet et al. 2008
	L. helveticus R0052, *B. longum* R0175	Reduced anxiety (murine model)	Messaoudi et al. 2010
	B. longum NCC3001	Decreased infection-induced behavioural changes (murine model)	Bercik et al. 2011

However, not all the probiotic strains drive a targeted therapeutic effect, thus, there is a need for rational selection of specific probiotic strains, matched for precise clinical indications. This implies that there is also a need to have a better insight in the effects that a specific probiotic strain can have in patient cohorts as well as in healthy populations. Furthermore, several studies demonstrated that the combination of more strains, also belonging to different genera (mainly Lactobacillus and Bifidobacterium), may exhibit a synergistic effect and research is therefore aimed at finding proper combinations.

26.5.1 Treatment and prevention of acute diarrhea and antibiotic-associated diarrhea

Infectious gastroenteritis is one of the leading cause of morbidity especially in newborns and children under 5 years of age (Wiegering et al. 2011). Although gastroenteritis-associated mortality is rare in Western Europe, an increased incidence has been noticed in some national registers over recent years. The most common causes of infant gatroenteritis are viruses (e.g. rotavirus, adenovirus and norovirus) and bacteria (e.g. *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *C. difficile*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *E. coli*), but parasite infections are also diffused. The vast majority of the published trials show a statistically significant benefit and clinical benefit of a few, well-identified probiotic strains. The effect is strain-dependent and dose-dependent. Positive outcomes in the treatment and prevention of diarrhea with probiotics date back in the '90s and the most studied strain was *L. rhamnosus* GG (LGG). LGG was particularly effective in the preventive therapy of diarrhea, whereas seriously sick children received less benefits from the treatment (Arvola et al. 1999).

The targeted effects of probiotics on viral diarrhea have been the subject of recent studies trying to elucidate the mechanisms of diarrhea symptoms relieve. In particular, in the study of Munoz et al. (2011) a *B. longum* susp. *infantis* strain, isolated from infant feces, was selected for the capability of inhibiting *in vitro* rotavirus replication and of protecting cells from virus infection. It was then tested *in vivo* on mice showing protection against rotavirus infection. A strong antiviral activity has been also obtained with *Lactobacillus ruminis* SPM0211 *in vitro* (Kang JY., et al 2013). Moreover, mixtures of probiotics showed good results in the reduction of duration and the severity of the acute rotavirus diarrhea. A multiple species product composed of a *L. acidophilus,* a *L. rhamnosus* and a *B. longum* strain, administered to infant aged 1-23 months, was able to reduce the duration of the disease (Grandy et al. 2010). Similar results have been obtained testing the efficacy of a synbiotic product named "Probiotical" containing *Streptoccoccus thermophilus, L. rhamnosus, L. acidophilus, B. animalis* subsp. *lactis, B. infantis* strains and fructooligosaccharides in children with acute diarrhea (Vandelplas and De Hert, 2011). The primary end-points were duration of diarrhea and the number of children that had a normalized stool consistency. A further study was aimed at evaluating the efficacy of administering *L. reuteri* DSM 17938 for the prevention of nosocomial diarrhea. In hospitalized children, the administration of *L. reuteri* DSM 17938 compared with placebo treatment had no effect on the overall incidence of nosocomial diarrhea, mainly caused by rotavirus infection (Wanke and Szajewska, 2012). Therefore, the studies on viral associated diarrhea still lack conclusive remarks.

Some probiotic strains resulted also effective in reducing the risk of antibiotic-associated diarrhea in newborns and children. A clinical trial, performed with 766 infants, indicated that treatment with the probiotic strain *Lactobacillus rhamnosus* GG), compared to placebo, reduced the risk of diarrhea from 28.5% to 11.9% (Szajewska et al. 2006). A number of study focus on the administration of probiotic in the treatment of diarrhea caused by bacteria, mainly *Clostridium* spp.. *Bifidobacterium* strains, deriving from infant feces and mainly belonging to the *B. breve* species, were capable of contrasting the growth of pathogens causing infectious diarrhea of bacterial origins in infants, making them potential probiotic candidates for a formulation aimed at the prevention or the cure of bacterial diarrhea (Aloisio et al. 2012). Furthermore, a recent study demonstrated the efficacy of *B. longum subsp. infantis* 35624 of reducing the effect of villi-associated enzyme caused by *Salmonella* infection (Symonds et al. 2012).

26.5.2 Treatment and prevention of necrotizing enterocolitis (NEC)

NEC is the most common gastrointestinal emergency in the neonatal intensive care unit and a major cause of morbidity in preterm infants. It is characterized by a gastrointestinal dysfunction progressing to pneumatosis intestinalis, systemic shock, and rapid death in severe cases. The most common risk factors are considered to be prematurity, enteral feeding and the occurring of bacterial colonization, which often operate in synergy (Claud and Walker, 2001). There is a strong evidence that the initial bacterial colonization after birth plays a pivotal role in the development of NEC. As already evidenced, preterm newborns show a different colonization with respect to full term newborns and more pathogenic microrganisms such as enterobacteria and enterococci remain predominant until the 20^{th} day of life. For this reason it has been suggested that a major etiological factor for NEC is a different microbiota, particularly as NEC usually occur after 8-9 days postpartum when anaerobic bacteria start to colonize the gut (Mai et al. 2011). This study also pointed out that correction of the abnormal microbiota composition may be a strategy to prevent NEC. Several authors have reported systematic reviews of randomized and controlled trials of probiotic supplementation in preterm infants (Deshpande et al. 2007; Barclay et al. 2007; Stockman 2009; Deshpande et al. 2010), showing that probiotic supplementation was able to reduce the incidence of NEC in neonates. The action attributed to probiotics is species specific (Verdu 2009). Administration of *B. bifidum, B. breve* and *B. animalis* susp. *lactis* strains to preterm and low birth weight infants showed evident clinical benefits for the treatment of NEC (Lin et al. 2008; Khailova et al. 2009; Braga et al. 2011; Underwood et al. 2012). The mechanisms by which probiotics operate are: competition against the colonization of potential pathogenic microorganism, immunomodulation, nutritional

contribution and improved intestinal motility. Treatment with *B. breve* associated with *L. casei* in preterm infants underlined a positive correlation between improved intestinal motility and NEC (Braga et al. 2011). On the contrary, *L. reuteri* does not seem to affect the overall rates of NEC and/or death in preterm infants followed up in the neonatal intensive care unit, and significant reductions were observed in the frequency of proven sepsis, rates of feeding intolerance and duration of hospital stay (Oncel et al. 2013). Because of the complexity of the pathogenesis of NEC, the administration of different *Bifidobacterium* and *Lactobacillus* strains may benefit by different actions on the host and, therefore, multistrain probiotic preparations are likely more effective than single-strain ones (Deshpande et al. 2011).

26.5.3 Prevention of infantile colics: are probiotics a feasible option?

About 10-30% of infants are affected by infantile colics in the first months of life, . The manifestations of colics are excessive, inconsolable crying, frequently accompanied by flushing of the face, meteorism, drawing-up of the legs and passing of gas. Microbiota of infants affected by colics was in fact found to be richer in gas forming coliforms with respect to non-colicky ones (Savino et al. 2009). Regarding possible therapies, the study of Savino et al. (2007) examined, for the first time, the modulation of intestinal microbiota of infants suffering from colics by administering a probiotic strain. A cohort of breastfed colicky infants was randomly assigned to treatment with a strain of *Lactobacillus reuteri* (ATCC 55730) and simethicone, an orally administered anti-foaming agent traditionally used to reduce discomfort or pain caused by excessive gas. This study evidenced that infants treated with *L. reuteri* had a significant reduction in crying compared to infants treated with simethicone. The positive effect of probiotic administration on the reduction of gas colic symptoms and on the modulation of intestinal microbiota was confirmed in a successive trial with the strain *L. reuteri* DSM 17938 (Savino et al. 2010). Moreover, the efficacy of the administration of the latter strain in reducing the daily average time of crying time was also confirmed by Szajewska et al. (2013). In addition, research is going on with the aim of obtaining new probiotic strains for possible use in colics treatment, such as other *Lactobacillus* strains (Savino et al. 2011) or *Bifidobacterium* strains. (Aloisio et al. 2012). The efficacy of probiotic supplementation in the reduction of crying time and successful treatment of infantile colic was the object of a recent systematic review (Anabrees et al. 2013). The final conclusion was that, although *L. reuteri* may be effective as a treatment strategy for crying in exclusively breastfed infants with colic, the evidence supporting probiotic use for the treatment of infant colic or crying in formula-fed infants remains unresolved. Larger rigorously designed studies are necessary to draw more definitive conclusions. Recently, a large clinical

trial on the efficacy of *L. reuteri* DSM 17938 in reducing the symptoms of infant colics obtained different results from previous trials (Sung et al. 2014). *L. reuteri* DSM 17938 administration to both breast-fed and formula fed newborns did not reduce crying or fussing. The efficacy of DSM 17938 and other probiotic strains should therefore be further explored.

26.5.4 Celiac disease (CD): *in vitro* studies need to supported by clinical trials

CD is a chronic inflammatory disorder of the small intestinal mucosa induced by the ingestion of wheat gluten, or other similar proteins found in barley and rye. The CD involves genetic and environmental factors in predisposed individuals (Green and Cellier 2007). CD is characterized by two types of immune response to gluten-derived peptides: an adaptive immune response Th_1-dependent, within the intestinal mucosa, and an innate immune response. Both responses lead to the release of proinflammatory cytokines like IFN-γ and IL-15 (Londei et al. 2005) and result in a consequent inflammation and intestinal tissue remodeling (Meresse et al. 2009). At present, the only effective treatment for the disease is a strict life-long gluten-free diet.

Palma et al. (2012) evaluated the gut microbial colonization during the first 4 months of life in breast-fed and formula fed healthy full-term infants with a genetic CD risk. They demonstrated that the milk-feeding type and the HLA-DQ genes (the major genetic risk factor for CD) influence the gut bacterial colonization. In particular, the reduction of *Bifidobacterium* spp. such as *B. longum* was associated with an increased CD risk. Until now relatively few studies considered the efficacy of the administration of probiotic strains in children affected by celiac disease. De Angelis et al. (2006) showed the capacity of the probiotic preparation VLS#3, a mixture of lactic acid bacteria and bifidobacteria (VSL Pharmaceuticals, Gaithesburg, MD) of decreasing the toxicity of gluten during food processing. Furthermore VLS#3 has been shown to colonize the intestine and increase epithelial barrier function in the host (Kaur et al. 2002) and it has the potential of being used to modify and improve gliadin degradation in the gastrointestinal tract after ingestion. Moreover a recent study investigated the capability of probiotic bacteria (*L. fermentum* and *B. lactis* strains) of inhibiting toxic effects induced by gliadin directly on epithelial cells (Lindfors et al. 2008). *B. lactis* resulted to be more efficient than *L. fermentum* in small harmless peptide products.

Therefore, although several studies have addressed the ability of probiotic bacteria to detoxify gliadin after an extensive incubation period and their role in suppressing proinflammatory effects *in vitro* to our knowledge no studies have investigated whether different live probiotic bacteria can inhibit gliadin-induced toxic effects directly on *in-vivo* epithelial cells.

26.5.5 Prevention of allergies

The incidence of allergic disorders such as atopic dermatitis, rhinitis and asthma has increased strikingly in developed countries. One of the most reliable hypotheses of this increase is a relative lack of microbial stimulation and a failure of immunoregulation due to low exposure of the infantile gut immune system to harmless microorganisms associated with the environment (Cabana et al. 2007). The manifestations of allergic disease are age dependent. Infants commonly present symptoms and signs of atopic eczema, gastrointestinal symptoms and recurrent wheezing. Asthma and rhinoconjunctivitis become prevalent in later childhood. Sensitization to food allergens usually occurs in the first two to three years of life, followed by indoor allergens (e.g. house dust mite and pets) and, subsequently, outdoor allergens (e.g. rye and timothy grass) (Halken 2004). Genetic susceptibility plays a large role in the development of food allergy, in fact the risk of development of allergy increases substantially with a positive family history of allergic diseases.The prevalence of allergic disease in childhood is 7% to 8% for food allergy, 15% to 20% for atopic eczema, and 31% to 34% for asthma or recurrent wheezing (Halken 2004). Food hypersensitivities affect approximately 6% of infants less than three years of age (Osterballe 2005). Although the exact etiology of allergic diseases remain ambiguous, epidemiological data have shown that atopic children show recurrent differences in the gut microbiota composition with respect to healthy children, with higher levels of clostridia and lower levels of bifidobacteria. In addition to lower number of bifidobacteria, infants suffering from atopic disease harbor a peculiar pattern of bifidobacteria comprising adult-like strains, such as *B. adolescentis*, as compared to healthy infants with a typical infant pattern (Ouwehand et al. 2001). Some other studies have also shown that early colonization with pathogenic bacteria is more likely to occur in children who go on to develop allergy; in contrast, lactobacilli and bifidobacteria are found more commonly in the composition of the gut microbiota of non-allergic children and this seems to correlate with protection against atopy (Kalliomaki et al. 2001; Ozdemir 2009). Therefore, the possibility of using probiotics to prevent the development of allergic disease has a sound scientific background.

A probiotic mixture, containing *B. breve* Bb99 strain, the LGG strain, *L rhamnosus* LC705 in addition to propionibacteria, was administered to mothers during the last month of pregnancy and their infants received it from birth until age 6 months. The treatment resulted in a decreased incidence of IgE-associated allergy, such as atopic diseases, in Cesarean-delivered children until the age of 5, with respect to the administration of a placebo (Kuitunen et al. 2009). The same probiotic mixture was also found to be effective in the treatment of eczema in infants with proven cow's milk allergy (Viljanen et al. 2005). Conversely, commercially available cow's milk

formula supplemented with *B. longum* BL999 and *L. rhamnosus* LPR administered in the first 6 months of life to Asian infants at risk of allergic disease showed no effect on prevention of eczema or allergen sensitization in the first year of life (Soh et al. 2009). The administration of *L. johnsonii* EM1 administered to 7-12 years old children was found to be more effective against allergic rhinitis than antihistamine treatment with levocetirizine (Lue et al., 2012). Other studies have been focused on the effects of probiotics in the treatment of food allergy, but conclusive effects have not been evidenced yet (Boyle and Tang 2006). Some preliminary positive results have been obtained with LGG supplementation, but no experimentation is available on bifidobacteria.

26.5.6 Obesity: a correlation with abnormal gut microbiota

Nowadays, obesity prevalence is increasing especially among children and adolescents and it can be considered a worldwide epidemic. Recently, obesity has been associated with structural alterations in the gut microbiota, suggesting potential causality between specific microbial taxa and this disorder. In particular, studies have focused not only on individual bacterial species but also on the whole microbial community (Tennyson and Friedman 2008). However, controversial data make it clear that the connection between the microbiota composition and excess weight is very complex. The explanations for the ability of the gut microorganisms to affect obesity development include an improvement of the energy harvest from the diet, influence on lipase activity, a decrease of lipopolysaccharide inflammation that is related to fat induced system and the control on endotoxemia and insulin resistance (Blaut and Bishoff 2010).

Million et al. (2012) found that bifidobacteria could associated to the lean status, on the contrary *Lactobacillus* spp., *Staphylococcus aureus* and *Escherichia coli* have been associated to overweight status. Members of the genus *Bifidobacterium* were shown to be higher in number in children who remained normal weight at 7 years old than in children developing overweight (Kalliomaki et al. 2008), allowing the authors to conclude that an aberrant compositional development of the gut microbiota precedes overweight and this may offer new possibilities for preventive and therapeutic applications of bifidobacteria in weight management. Furthermore, other studies reported a decrease of total bifidobacteria in feces of obese patients (Schwiertz at al. 2010; Collado et al. 2008). A recent study analyzed the fecal concentration of the main intestinal microbial groups in obese, overweight, lean and anorexic subjects. A positive correlation was found between certain *Lactobacillus* species, in particular *L. reuteri*, and obesity. On the contrary, *B. animalis* has been associated with a lower Body Mass Index (Million et al. 2013). Several meta-analyses, in the past years, have shown that breastfeeding is associated with

a reduced likelihood of overweight or obesity in childhood and that the duration of breastfeeding is inversely associated with the risk of overweight (Owen et al. 2005; Harder et al. 2005). Several results mainly performed on murine models suggested that some strains of bifidobacteria and lactobacilli have effect on the obese status reducing serum total cholesterol, decreasing proinflammatory cytokines and increasing glucose tolerance (Cani et al. 2007, Ma et al. 2008, Yin et al.2010, An et al. 2011). However, the *in vivo* administration of probiotics in obese children or adults needs to be investigated.

26.5.7 Treatment of neurological and psychiatric disease

The gut and the brain are highly integrated and communicate in a bidirectional manner and this connection is usually called "the gut-brain axis" (Rhee et al. 2009). An example of this relation is that psychiatric disorders frequently coexist with common pathological gastrointestinal conditions, such as irritable bowel syndrome, which is quite diffused in adults. The gastrointestinal tract is a site of interaction between microorganisms, immune cells, and the neuronal network. In this respect, beneficial microbes such as lactobacilli and bifidobacteria seem to be particularly sensitive to signals from the central nervous system, taking into consideration that stressful conditions, including emotional stress, are very often accompanied by a decrease of these organisms in the gastrointestinal tract. Neurochemical molecules can affect the microbiota composition chancing the gut motility and increasing the acidity. Moreover, bacteria themselves can influence the endocrine system by the production of several biologically active peptides, nitric oxide, melatonin, gamma-aminobutiryc acid and serotonin (Collins and Bercik 2009). Pro-inflammatory cytokine such as IL4 and interferon γ are implicated in a range of psychiatric disorders including depression and studies in animals and humans have showns that manipulation of the gut microbial composition influences systemic cytokine levels (Cryan and O'Mahoni 2011). A decrease in the desirable gastrointestinal tract bacteria will lead to deterioration in gastrointestinal, neuroendocrine and immune relationships and, ultimately, disease. Therefore, studies focusing on the impact of enteric microbiota on the central nervous system are essential to the understanding of the influence of this system.

A possible approach to study the microbiota-gut-brain axis is the use of germ-free mice. Researches performed with acute stressed germ free mice, showing hyperactivity of the body major stress response system (the hypothalamic-pituitary-adrenal axis), have evidenced that the stress response was normalized by administration of a *B. infantis* strain (Sudo et al. 2004). Bercik et al. (2011) showed that infection-induced behavioral changes in mice could be reversed by *B. longum* NCC3001 strain administration. *B. infantis* 35624 strain has been shown in

Sprague-Dawley rats to induce an increase of levels of plasma tryptophan, a precursor of serotonin which is a key neurotransmitter within the gut-brain axis possessing antidepressant properties (Desbonnet et al. 2008).

The research in the neurology and psychiatry sector has scarcely reached the point of intervention studies targeted to humans and, in particular, to infants. However, probiotics may offer a potential therapeutic that could beneficially alter the gut-brain axis and modify aberrant behaviors in infant related to altered immune inflammatory outputs and such as autism spectrum disorders. One of the few intervention studies performed has assessed the effect of a combination of *Lactobacillus helveticus* R0052 strain and *B. longum* R0175 strain on both human subjects and rats showing that these probiotics reduced anxiety in animals and had beneficial psychological effects with a decrease in serum cortisol in patients (Messaoudi et al. 2010). Moreover a study on human neonates showed that the pattern of electrical activity in the brain is less complex in neonates born by caesarian section than in age matched neonates born by vaginal delivery. These results raise the possibility that different colonization patterns influence early post natal brain development and also have longer term consequences (Kim et al. 2003). The aim for future research in this field is to definitely clarify the effects of the gut microbiota on several brain-related functions in order to identify the microbial species that are critical for the development of a healthy phenotype and those that may have negative impacts on behavior, mood and emotion in humans. This will pose the basis for targeted probiotic intervention trials.

26.6 Prebiotics and their pediatric use

Prebiotics are non-digestable food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon, that can improve the host health (Roberfroid 2007). They are low molecular weight carbohydrates, mainly oligosaccharides (i.e. composed of three to ten monomers linked together), mostly constituted of fructose, glucose or galactose. Prebiotics resist gastric acidity, are hardly hydrolyzed by human enzymes and are not absorbed in the upper gastro-intestinal tract. They reach the colon virtually intact and stimulate the colonic microbiota, resulting in growth and fermentation products influencing the host immunity, metabolism and mineral absorption (Roberfroid et al 2010). The indigestibility of these molecules in the upper gastro-intestinal tract has allowed their definition as Non Digestible Oligosaccharides (NDOs). It is therefore true that prebiotics allow specific changes both in the composition and/or activity of the intestinal microbiota, which results in nutritional benefits (Saulnier et al. 2009) and positive aspects to human health, as already evidenced in Chapter 21-23 of this book. Beneficial bacteria

play useful roles in aspects of nutrition and prevention of disease. The largest part of the species belongs to the genera *Lactobacillus* and *Bifidobacterium*. NDOs entering the colon are fermented selectively by members of these genera capable of producing glycolytic enzymes, so they are hydrolyzed into mono- or disaccharides which can be transported into the cell where they can be metabolized into short chain fatty acids (SCFAs). SCFAs and their metabolites are responsible for a large array of beneficial NDOs effects (Roberfroid et al 2010). The ability to utilize a large variety of oligosaccharides by bifidobacteria makes them able to adapt and compete in an environment with changing nutritional conditions. For this reason bifidobacteria are considered one of the most efficient groups at utilizing NDOs allowing them to proliferate with respect to other species when probiotics are consumed. The particularly important role that bifidobacteria exert in the infant gut makes the use of prebiotics very important in infant nutrition, in particular for those infants who are not fed by maternal milk. The main characteristics of the most common NDOs used in infants are presented in Table 3.

Table 3. Properties of non-digestible oligosaccharides mainly used in infants (see following section for studies regarding their use).

Name	Structure	Method of manufacture	Polymerization Degree
Galactoologosaccharides	(Gal)n-Glu	Synthesis from lactose by β-galactosisase	2-8
Fructooligosaccharides	(Fru)n-Glu	Synthesis from sucrose or hydrolysis of inulin	2-10
Polydextrose	(Glu)n-Sor	Synthesis from glucose	10-12
Lactulose	Gal-Fru	Isomerization of lactose.	2
Acidic oligosaccharides	Mixture of linear oligomers and small polymers of GalA with a 50% methylation degree	Enzymatic digestion of food grade pectin	n.a.

Gal: galactose; Glu: glucose; Fru: fructose; Sor: sorbitol; GalA: galacturonic acid

26.6 .1 Prebiotic activity of human milk oligosaccharides

The peculiar composition of the intestinal microbiota of breast-fed neonates previously outlined is in part due to the presence of complex molecules possessing prebiotic effects in human milk. The most abundant molecules with this role are oligosaccharides, referred to as human milk oligosaccharides (HMOs). HMOs are resistant to digestive processes and thereby reach the colon, where they can be digested by intestinal bacteria, suggesting they have a particular prebiotic role (Arslanoglu et al. 2007). Because of the intensive interaction between the intestinal microbiota and the epithelium as well as the intestinal immune cells, this prebiotic effect is crucial for the expansion and education of the immune system early in life (Schouten et al. 2011). HMOs are synthesized in the mammary gland by the action of specific glycosyltransferases by the sequential addition of monosaccharide units to the lactose molecule. With few exceptions, all known HMOs have a lactose core and are elongated via linkage to one or more units of galactose and N-acetylglucosammine and can be decorated with several fucose and sialic acid residues. Because of the possibilities for different backbone length and decoration with different combinations of the basic building blocks, many HMO structures exist (Pfenninger et al. 2002). An example of HMO configuration is depicted in Figure 1.

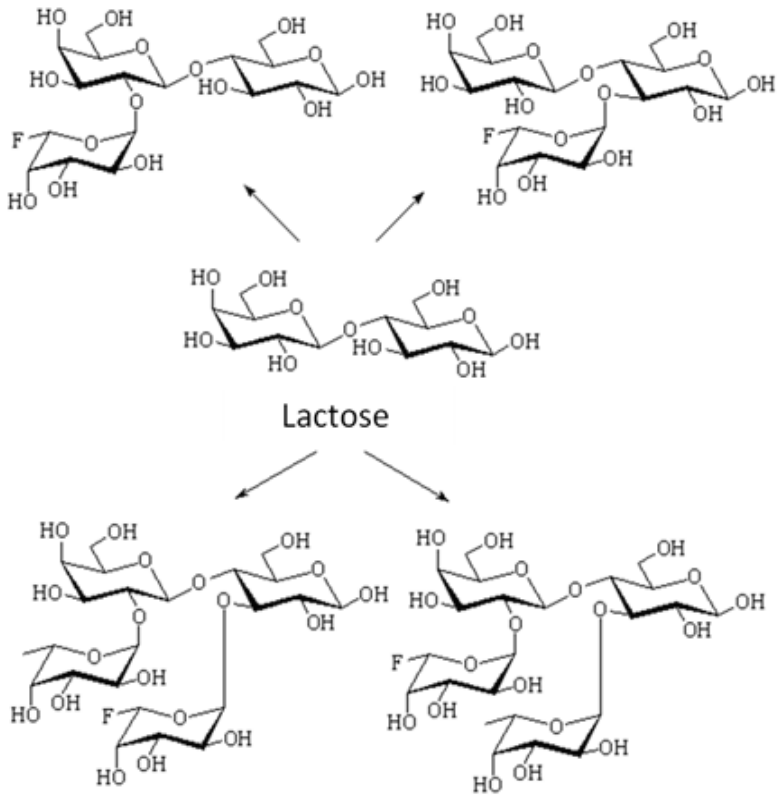

Figure 1 Possible chemical structure of human milk oligosaccharides. F represents fucose residues bound to lactose backbone.

Over the years the prebiotic effect of HMOs has been confirmed. *In vitro* fermentation studies clearly demonstrated that the bifidogenic effect of maternal milk is mainly due to the "non-protein fraction" and that HMOs have a pivotal role in stimulating the selective development of bifidobacteria (Ward et al. 2006). In this study, it has been demonstrated that *B. infantis* is able to use complex carbohydrates such as HMOs as the sole source of carbon and energy.

Recent studies focused on the molecular mechanisms underlying the role of human milk on the development of intestinal bifidobacterial community. The identification of genes expressed by *B. breve* strains, upon HMO stimulation, represented the preliminary insight to understand the molecular mechanisms governing the initial stages of bacterial colonization in newborns (Turroni et al. 2012). Human milk can also be the direct source of beneficial bacteria: some papers reported the isolation of bifidobacteria from human milk (Martin et al. 2003), although it is also possible that bifidobacteria are introduced into human milk through newborn-mother contacts. Another characteristic substance of human milk is lactoferrin, a glycoprotein member of the transferrin family. In human milk it is the most abundant protein, on the contrary it is present only in traces in cow's milk. A small percentage of lactoferrin (about 6% to 10%) is estimated not to be digested by breast-fed infants, it could consequently reach the colon and play a role as a prebiotic. The availability of bovine lactoferrin has made it possible to add lactoferrin to infant formulas and to study the effect of feeding such formulas to infants. Recent studies have been found that lactoferrin appears to exert a prebiotic effect but an addition of lactoferrin in formula has a little effect on the newborn fecal microbiota (Coppa et al. 2006).

Other groups of substances studied for their possible prebiotic role are nucleotides. Human milk contains high concentrations of preformed nucleotides, whereas cow's milk is usually devoid of such compounds. Some studies have also suggested a prebiotic role for lactose as it has been demonstrated that lactose reaching the colon stimulates the growth of bifidobacteria, although the amount of ingested lactose reaching a neonate's colon is very low,(Szilagyi et al. 2002). Is it also true that a certain amount of lactose could remain after the fermentation by the intestinal microbiota and could be metabolized by bifidobacteria (Parche et al. 2006). In particular, studies have demonstrated that *B. longum* exhibits a preferential metabolic pathway for the use of lactose. In addition, bifidobacteria possess several homologous genes encoding enzymes which are involved in the metabolism and transport of numerous sugars.

26.6 .2 Formula milk enriched with prebiotics: are they comparable to human milk?

Cow's milk, which is commonly used in the preparation of infant milk formulas, and human milk have significant differences. HMOs are one of the most important components in human

milk, in contrast, these oligosaccharides are present only in small amounts in cow's milk. Within the complex mechanism that regulate the development of the intestinal microbiota, the ability to utilize complex carbohydrates is believed to exert an important influence on the development of specific bacteria strains over others; in the gastrointestinal tract of breast-fed neonates, the relationship between HMOs and the development of bifidobacteria represents a typical example of this situation. For this reason, prebiotic oligosaccharides, in particular galactooligosaccharides (GOS), are abundantly used in infant formula (Coppa et al. 2006; Boehm et al. 2007). The role of these molecules in newborns as an additive to cow milk started to the studied from the beginning of the XXI century and, since then, several achievements have been reached. They are described in details below and briefly summarized in Table 4.

Table 4 Positive effects upon administration of formula milk enriched with prebiotics.

Prebiotics added to formula milk	**Total prebiotic concentration in the formula milk**	**Detected positive effects**	**Reference**
scGOS/ lcFOS 9:1	0.4 % and 0.8 % (w/v)	Increase in bifidobacteria and lactobacilli content	Moro et al. (2002)
		Softer stools	
		No side effects	
scGOS/ lcFOS, 9:1	0.8 % (w/v)	Pathogen reduction in stools	Boehm et al. (2004)
scGOS /lcFOS, 9:1	0.6 % (w/v)	Metabolic activity of the microbiota similar to that of breast-fed infants	Bakker-Zierikzee et al. (2005)
		Increased saccharolytic activity in the colon with respect to proteolytic activity	
scGOS/lcFOS, 9:1	0.8 % (w/v)	Fewer infectious episodes	Arslanoglu et al. (2007)
		Fewer number of infections requiring antibiotics	
		Reduced incidence of infections	
PDX /GOS, 1:1	0.4% (w/v)	Consistency of stools similar to breast-fed infants	Ziegler et al. (2007)
PDX/GOS/LOS, 0.50:0.33:0.17	0.8 % (w/v)	Consistency of stools similar to breast-fed infants	Ziegler et al. (2007)
PDX /GOS, 1:1	0.4% (w/v)	Gut microbiota composition similar to breast-fed infants	Salminen et al. (2014)

A preliminary study (Boehm et al. 2000) checked the prebiotic capacity of an oligosaccharide mixture consisting of a 9:1 mixture of short chain (sc)-GOS (derived from lactose) and long chain (lc)-fructooligosaccharide (FOS) (high-molecular-weight fraction of inulin extracted from chicory roots) on pre-term infants. The mixture was designed to mimic the molecular size distribution of HMO and was used at a concentration of 1 % (w/v), similar to the oligosaccharide content of human milk, to enrich the composition of a formula-milk. The result of this study showed that infants fed with this formula had a bifidobacteria content that was in the upper range of the values found in infants fed human milk. The same oligosaccharide-supplemented formula milk was assayed in healthy term infants, by using two different concentrations of oligosaccharides (0.4 % and 0.8%, w/v) (Moro et al. 2002). After 28-day feeding period, the number of *Bifidobacteria* was significantly increased in both groups receiving supplemented formulas with respect to the control group (fed with a formula milk not enriched with prebiotics) and the effect was dose dependent. The number of lactobacilli also increased significantly in both groups fed the supplemented formulas, but there was no dose dependent effect. Supplementation had a significant dose dependent influence on stool consistency, which was softer in prebiotic supplemented newborns, whereas it had no influence on the incidence of side effects (crying, regurgitation, vomiting) or growth. A successive study using the same enriched formula milk with FOS-GOS at 0.8 % (w/v) allowed to reach further achievements: not only was the number of beneficial bacteria increased, but also the number of potential pathogens was reduced in supplemented milk fed newborns compared to the control group (Boehm et al. 2004). Moreover, the amount of bifidobacteria was similar to that typical of breast-fed infants. *In vitro*, the short-chain fatty acids produced by the FOS-GOS mixture were similar to those produced by the HMO fraction. In the clinical trials, the pattern of fecal short-chain fatty acids of infants fed the oligosaccharide mixture was similar to that of breast-fed infants but was significantly different from that of a group of infants fed with an unsupplemented formula.

Bakker-Zierikzee et al. (2005) compared the effects of two infant formulas, one containing a mixture of GOS and FOS 9:1 (0.6% w/v) and the other containing the probiotic strain *Bifidobacterium animalis* Bb-12 (6.0×10^{10} CFU/l), on the composition and metabolic activity of the intestinal bacteria after 4 months of administration. The control group received a non-supplemented standard formula and a group of breast-fed infants was also included as a reference group. Compared with the groups fed Bb-12 and standard formula, the GOS/FOS formula group showed higher faecal acetate ratio and lactate concentration, allowing to conclude that the metabolic activity of the microbiota in this group is similar to that of breast-fed infants. The differences in the short-chain fatty acids observed in the GOS/FOS group are consistent with

a shift from a more proteolytic/putrefactive to a more saccharolytic colon physiology, which can be considered a health benefit for the infant. Differences in bifidobacteria counts between the GOS/FOS, Bb-12 and the standard groups were not statistically significant.

A mixture of short chain GOS and long chain FOS (scGOS/lcFOS, 0.8% w/v) has been shown to have prebiotic and immunomodulatory effects comparable HMO in healthy term infants with a parental history of atopy (Arslanoglu et al. 2007). Although these oligosaccharides are not identical to HMO, studies in preterm and term infants have shown that a formula supplementation with this prebiotic scGOS/lcFOS mixture results in an intestinal microbiota similar to that found in breast-fed infants (Knol et al. 2005; Boehm et al. 2002). Infants supplemented with the scGOS/lcFOS formula milk had fewer infectious episodes, in particular respiratory infections, fewer number of infections requiring antibiotics, and a reduced incidence of infections during the first 6 month of life.

Other studies explored the use of different prebiotic mixtures. Ziegler et al. (2007) studied the effect of different combinations of polydextrose (PDX), GOS, and lactulose (LOS), on the overall growth and tolerance in healthy term infants up to 120 days of age. Beside the control group fed with a standard formula, other two groups were fed with a control formula supplemented with 0.4% (w/v) of a prebiotic blend containing PDX and GOS, 1:1 and with a control formula supplemented with 0.8 % of a prebiotic blend containing PDX, GOS, and LOS, 0.50:0.33:0.17. There were no statistically significant differences among the 3 formula groups for weight growth rate or length growth rate. The supplemented formula groups had looser stools, more similar to those of breast-fed infants, than the control group. However, a slight increase in the eczema frequency was observed when newborns were fed with the prebiotic blend containing PDX and GOS.

A double-blind, randomized, prospective study, healthy term infants vaginally born and exclusively formula-fed received a standard cow's milk-based formula or the same formula added with PDX and GOS (1:1 ratio), 0.4 % (w/v). A reference breastfed group was included. The study allowed to conclude that modifying formula feeding by adding prebiotics may bring the gut microbiota closer to that of breast-fed infants (Salminen et al. 2014).

26.6.3 Prebiotics in infants for prevention of allergy

As already pointed out in section 5.4, food allergy and allergic disease represent a substantial health problem that is increasing in children. . Among other factors, an altered microbial exposure in the gastrointestinal tract may be partly responsible for the increase of allergic diseases in populations with a western lifestyle. The gastrointestinal microbiota modulates

mucosal physiology, barrier function and systemic immunologic and inflammatory responses. The efficiency of this gastrointestinal barrier is reduced in the newborn period and it can make newborns more susceptible to allergies. The composition of the intestinal microbiota is different in infants with atopic eczema, in particular a reduced bifidobacteria content has been shown in infants with eczema and atopic sensitization (Osborn and Sinn 2013). Moreover, such differences may precede the development of eczema. The recognition of the importance of intestinal biota has led to the development of strategies aimed at manipulating bacterial colonization in formula fed infants, including the use of prebiotics.

Among the several studies present in the literature, Osborn and Sinn (2013) considered four of them as the most convincing in the field of prebiotic use in infants to prevent allergies. The outcomes are briefly summarized in Table 5.

Table 5 Detected effects on the risk of allergies upon administration of formula milk enriched with prebiotics.

Target of the study	Prebiotics used	Detected effects	Reference
Infants with high risk of eczema development	GOS/ FOS 9:1 (0.8 % w/v)	Reduction in the incidence of dermatitis	Moro et al. (2006)
Infants with no specified risk of eczema development	PDX /GOS, 1:1 (0.4% w/v)	Slight increase in the eczema frequency	Ziegler et al. (2007)
Infants with low atopy risk	GOS/ FOS (9:1) plus acidic oligosaccharides (0.8 % w/v)	Positive effect in the prevention of atopic dermatitis The preventive effect persisted beyond the first birthday A reduced incidence of respiratory allergy later in life.	Gruber et al. (2010)
Preterm infants	Mixture of neutral and acidic oligosaccharides	No reduction in the incidence of allergic diseases during the first year of life	Westerbeek et al. (2010); Westerbeek et al. (2013)

In the study of Moro et al. (2006) infants possessing an high risk of eczema development were fed with an hydrolysed whey protein formula supplemented with a mixture of FOS and GOS (0.8 % w/v) versus the same formula with added maltodextrin at the same concentration. The incidence of development of the dermatitis was reduced in the GOS-FOS administered group and it was associated with a significantly higher number of bifidobacteria compared with controls; conversely, no significant difference in lactobacilli counts was detected. This study showed for

the first time a beneficial effect of prebiotics on the development of atopic dermatitis in a high risk population of infants. Although the mechanism of this effect was not fully elucidated, it appeared likely that oligosaccharides modulate postnatal immune development by altering bowel microbiota and had a potential role in primary allergy prevention during infancy. On the other hand, a different achievement was reported in the study of Ziegler et al. (2007), already described in the previous section. Although the authors reported a slight increase in the eczema frequency when newborns were fed with the prebiotic blend containing PDX and GOS, this study did not enroll infants with a high risk of development of eczema. Gruber et al. (2010) recruited infants with low atopy risk before the age of 8 weeks to receive a regular cow's milk formula with added neutral GOS and FOS (9:1) and acidic oligosaccharides (OS) (total 0.8 %) versus a control group who received cow's milk based formula without added oligosaccharides. The main outcome of this study was that formula supplementation with a specific mixture of oligosaccharides was effective as primary prevention of atopic dermatitis. The authors speculated that the effect persisted beyond the first birthday and might even result in a reduced incidence of respiratory allergy later in life.

Other studies were aimed at determining the effect of short-term enteral supplementation of neutral and acidic oligosaccharides during the neonatal period in preterm infants on the incidence of allergic and infectious diseases during the first year of life (Westerbeek et al. 2010 and 2013). A group of newborns received enteral neutral and acidic oligosaccharides supplementation or placebo (maltodextrin) between day 3 and 30 of life. It was concluded that short-term enteral supplementation of a prebiotic mixture of neutral and acidic oligosaccharides during the neonatal period in preterm infants did not decrease the incidence of allergic and infectious diseases during the first year of life. Moreover, enteral supplementation of the prebiotic mixture did not significantly reduce the risk of serious infectious morbidity in preterm infants, but there was a trend toward a lower incidence of serious infectious morbidity, especially for infections with endogenous bacteria (Westerbeek et al. 2010). This was ascribed to an increase of postnatal intestinal colonization (Westerbeek et al. 2013).

26.7 Conclusion and future trends

A critical examination of the results present in the literature allows to conclude that the research on probiotics and prebiotics is currently very active and a lot of results on their efficacy are at present available. This hectic research activity has been stimulated by the evidence, acquired mainly in the last twenty years and supported by recent findings obtained via next generation molecular techniques, that the correlation between the microbiota composition and sickness

exists for several diseases both interesting the gut system and not strictly enteric. Research has also allowed to conclude that the efficacy of probiotics for the treatment and prevention of a target diseases is strain specific and that not only the capability of colonizing the gut is important, but also the production of anti-inflammatory molecules and the stimulation of the gut immune system and the systemic immune system. Gaining new experimental results is particularly important for the research field interesting children, because they represent an interesting target for industry and because non chemoterapic treatments are particularly looked forward for them.

Extremely relevant are the results obtained to make bottle nutrition more similar to breast feeding with prebiotic supplementation. These studies are all in agreement in stating that prebiotic enriched formula makes the gut microbial composition of formula fed infants similar to that of breast-fed newborns. On the contrary, the research on the potentiality of prebiotics to stimulate the beneficial colonic microbiota in case of disease is still sparse and mainly focused on allergic diseases. It is foreseeable that the opportunity of using the prebiotic strategy to prevent and reduce the symptoms of diseases such as celiac disease, obesity and neurologic upset is explored in future research activities, also considering that no adverse effects have been associated with prebiotic administration in newborn and infants.

Thererfore, children are a very important target, if not the main one, for probiotic and prebiotic administration and the European industry is aware of that.

Bibliography

Aloisio I, Santini C, Biavati B, Dinelli G, Cencič A, Chingwaru W, Mogna L, Di Gioia D (2012) Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns. Appl Microbiol Biotechnol 96:1561-1576

An HM, Park SY, Lee do K, Kim JR, Cha MK, Lee SW, Lim HT, Kim KJ, Ha NJ (2011) Antiobesity and lipid-lowering effects of *Bifidobacterium* spp. in high fat diet-induced obese rats. Lipids Health Dis 10:116

Anabrees J, Indrio F, Paes B, Afaleh K (2013) Probiotics for infantiles colic: a systematic review BMC 13:186-195

Arslanoglu S, Moro GE, Boehm G (2007) Early Supplementation of Prebiotic Oligosaccharides Protects Formula-Fed Infants against Infections during the First 6 Months of Life. J Nutr 137:2420–2424

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto J-M, Bertalan M, Borruel N, Casellas F, et al., (2011) Enterotypes of the human gut microbiome. Nature 473:174–8

Arvola T, Laiho K, Torkkeli S, Mykkanen H, Salminen S, Maunula L, Isolauri E (1999) Prophylactic Lactobacillus GGReduces Antibiotic-Associated Diarrhea in Children With Respiratory Infections: A Randomized Study. Pediatrics 104:e64

Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJM, Bindels JG (2005) Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacterium animalis* on the intestinal microflora during the first 4 months of life. Br J Nutr 94:783–90

Barclay AR, Stenson B, Simpson JH, Weaver LT, Wilson DC (2007) Probiotics for necrotizing enterocolitis: a systematic review. J Pediatr Gastroenterol Nutr 45:569-576

Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett P, Fahnestock M, Moine D, Berger B, et al., (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut–brain communication. Neurogastroenterol Motil 23:1132-1139

Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C (2010) Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev 86:13-15

Biavati B, Castagnoli P, Crociani F, Trovatelli LD (1984) Species of the *Bifidobacterium* in the feces of infants. Microbiol 7:341-345

Blaut M, Bischoff SC (2010) Probiotics and obesity. Ann Nutr Metab 57:20-23

Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, Marini A (2002) Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. Arch Dis Child Fetal Neonatal Ed 86:F178–81

Boehm G1, Jelinek J, Stahl B, van Laere K, Knol J, Fanaro S, Moro G, Vigi V (2004) Prebiotics in infant formulas. J Clin Gastroenterol38:76-79

Boehm G, Stahl B, Jelinek J, Knol J, Miniello V, Moro GE (2007) Prebiotic carbohydrates in human milk and formulas. Acta Paediatr 94:18–21

Boyle RJ, Tang ML (2006) The role of probiotics in the management of allergic diseases. Clin Exp Allergy 36:568-576

Braga TD, da Silva GA, de Lira PI, de Carvalho Lima M (2011) Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. Am J Clin Nutr 93:81-86

Cabana MD, McKean M, Wong AR, Chao C, Caughey AB (2007) Examining the hygiene hypothesis: the trial of infant probiotic supplementation. Paediatr Perinat Epidemiol 21:23-28

Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM (2007) Selective increases of bifidobacteria in gut microflora improves high-fat diet induced diabetes in mice through a mechanism associated with endotoxemia. Diabetolog 50:2374-2383

Cencič A., Langerholc T. (2010) Functional cell models of the gut and their applications in food microbiology-a review. Int J Food Microbiol 141*:* 4-14.

Chen C-C, Walker WA (2011) Clinical applications of probiotics in gastrointestinal disorders in children. 24: 153-160

Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, et al., (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 108 Suppl :4586–91

Claud EC, Walker WA (2001) Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. FASEB J 15:1398-1403

Collado MC, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y (2008) Imbalances in faecal and duodenal *Bifidobacterium* species composition in active and non-active celiac disease. BMC Microbiol 8:232

Collins SM, Bercik P (2009) The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterol 136:2003-2014

Coppa G V, Zampini L, Galeazzi T, Gabrielli O (2006) Prebiotics in human milk: a review. Dig Liver Dis 38 Suppl 2:S291–294

Coppa G, Gabrielli O (2008) Human milk oligosaccharides as prebiotics. In: Versalovic, J. and Wilson, M. (eds.) Therapeutic microbiology: probiotics and related strategies. American Society for Microbiology Press, Washington, pp 131-146

Cryan JF, O'Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behavior. Neurogastr Motil 23:187-192

De Angelis M, Rizzello CG, Fasano A Clemente MG, De Simone C, Silano M, De Vincenzi M, Losito I, Gobbetti M (2006) Vsl#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for Celiac Sprue. Biochim Biophys Acta 1762:80-93

Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan T (2008) The probiotic *Bifidobacterium infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 43:164-174

Deshpande G, Rao S, Patole S (2007) Probiotics for prevention of necrotizing enterocolitis in preterm neonates with very low birthweight: a systematic review of randomized controlled trials. Lancet 369:1614-1620

Deshpande G, Rao S, Patole S, Bulsara M (2010) Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics 125:921-930

Deshpande GC, Rao SC, Keil AD, Patole SK (2011) Evidence-based guidelines for use of probiotics in preterm neonates. BMC Med 9:92

Di Gioia D, Aloisio I, Mazzola G, Biavati B (2014) Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. Appl Microbiol Biotechnol 98:563–577

Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, Aguilera M, Khanna S, Gil A, Edwards CA, Doré J, INFABIO team (2010) Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr 51:77-84

Grandy G, Medina M, Soria R, Terán CG, Araya M (2010) Probiotics in the treatment of acute rotavirus diarrhoea. A randomized, double-blind, controlled trial using two different probiotic preparations in Bolivian children. BMC Infect Dis 10:253

Green PH, Cellier C (2007) Celiac disease. N Engl J Med 357:1731-1743

Grüber C, van Stuijvenberg M, Mosca F, Moro G, Chirico G, Braegger CP, Riedler J, Boehm G, Wahn U (2010) Reduced occurrence of early atopic dermatitis because of immunoactive prebiotics among low-atopy-risk infants. J Allergy Clin Immunol 126:791–797

Haarman M., Knol J. (2005) Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 71: 2318-2324.

Halken S. (2004) Prevention of allergic disease in childhood: clinical and epidemiological aspects of primary and secondary allergy prevention. Pediatr Allergy Immu 15 Suppl 16:4–5

Harder T, Bergmann R, Kallischnigg G, Plagemann A (2005) Duration of breastfeeding and risk of overweight: a meta-analysis. Am J Epidemiol 162:397-403

Jankowska A., Laubitz D., Antushevich H., Zabielski R., Grzesiuk E. (2008) Competition of Lactobacillus paracasei with Salmonella enterica for adhesion to Caco-2 cells. J Biomed Biotechnol 59:795-810.

Kalliomaki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E (2001) Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J Allergy Clin Immunol 107:129-134.

Kang C, Gayen S, Wang W, Severin R, Chen AS, Lim HA, Chia CSB, Schüller A, Doan DNP, Poulsen A, Hill J, Vasudevan SG, Keller TH (2013) Exploring the binding of peptidic West Nile virus NS2B-NS3 protease inhibitors by NMR. Antiviral Res 97:137-44

Kaur IP, Chopra K, Saini A (2002) Probiotics: potential pharmaceutical applications. Eur J Pharm Sci 15:1-9

Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M, Dvorak B (2009) *Bifidobacterium bifidum* improves intestinal integrity in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointes Liver Physio 1297:940-949

Kim HR, Jung YK, Kim SY, Ko KO, Lee YM, Kim JM (2003). Delivery modes and neonatal EEG: spatial pattern analysis. Early Hum De. 75: 35–53

Klaassens ES, Boesten RJ, Haarman M, Knol J, Schuren FH, Vaughan EE, de Vos WM (2009) Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants. Appl Environ Microbiol 75:2668-2676

Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, Klarczyk M, Schöpfer H, Böckler H-M, Wells J (2005) Colon microflora in infants fed formula with galacto- and fructooligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr 40:36–42

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE (2011) Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci USA 108:4578-4585

Kuitunen M, Kukkonen K, Juntunen-Backman K, Korpela R, Poussae T, Tuure T, Haahtela T, Savilahti E (2009) Probiotics prevent IgE-associated allergy until age 5 years in caesarean delivered children but not in the total cohort. J Allergy Clin Immunol 123:335-341

Laux D., Cohen P., Coneay T.(2005) Role of the mucus layer in bacterial colonization of the intestine. In Nataro J., Cohen P., Mobley H. and Weiser J.Colonization of the mucosa surfaces. American Society for Microbiology Press, Washington, pp 199-212

Ley R., Peterson D., Gordon J. (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124: 837-848

Lin HC, Hsu CH, Chen HL, Chung MY, Hsu JF, Lien RI, Tsao LY, Chen CH, Su BH (2008) Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. Pediatrics 122:693-700

Lin HC, Hsu CH, Chen HL, Chung MY, Hsu JF, Lien RI, Tsao LY, Chen CH, Su BH (2008) Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. Pediatr 122:693-700

Lindfors K, Blomqvist T, Juuti-Uusitalo K, Stenman S, Venäläinen J, Mäki M, Kaukinen K (2008) Live probiotic *Bifidobacterium lactis* bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. Clin Exp Immunol 152:552-558

Londei M, Ciacci C, Ricciardelli I, Vacca L, Quaratino S, Maiuri L (2005) Gliadin as a stimulator of innate responses in celiac disease. Mol Immunol 42:913-918

Lue KH, Sun HL, Lu KH, Ku MS, Sheu JN, Chan (2012) *Lactobacillus johnsonii* EM1 to levocetirine for treatment of perennial allergic rhinitis in children aged 7-12 years. Int J Pedriatr Otorinolaryngol 76: 994-1001

Ma X, Hua J, Li Z (2008) Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. J Hepatol 49:821-830

Magne F, Hachelaf W, Suau A, Boudraa G, Mangin I, Touhami M, Bouziane-Nedjadi K, Pochart P (2006) A longitudinal study of infant faecal microbiota during weaning. FEMS Microbiol Ecol 58:563-571

Magne F, Hachelaf W, Suau A, Boudraa G, Mangin I, Touhami M, Bouziane-Nedjadi K, Pochart P (2006) A longitudinal study of infant faecal microbiota during weaning. FEMS Microbiol Ecol 58:563-571

Mai V, Young CM, Ukhanova M, Wang X, Sun Y, Casella G, Theriaque D, Li N, Sharma R, Hudak M, Neu J (2011) Fecal microbiota in premature infants prior to necrotizing enterocolitis. PLoS One 6:e20647

Martin R., Langa S., Reviriego C., Jimenez E., Marin M., Xaus J., Fernandez J., Rodriguez J., (2003) Human milk is a source of lactic acid bacteria for infant gut. J Pediatr 143:754-758.

Meresse B, Ripoche J, Heyman M, Cerf-Bensussan N (2009) Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. Mucosal Immunol 2:8-23

Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejedi A, Bisson J, Roujeot C, PichelIn M, Cazaubiel M, Cazaubiel J (2010) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 26:1-9

Million M, Angelakis E, Maraninchi M, Henry M, Giorgi R, Valero R, Vialettes B, Raoult D (2013) Correlation between body mass index and gut concentrations of *Lactobacillus reuteri*, *Bifidobacterium animalis*, *Methanobrevibacter smithii* and *Escherichia coli*. Int J Obes 1-7

Million M, Maraninchi M, Henry F, Armougom F, Richet Carrieri H, Valero R, Raccah D, Viallettes B, Raoult D (2012) Obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* and depleted in *Bifidobacterium animalis* and *Methanobrevibacter smithii* Int J Obes 36:817-825

Montier Y., Lorentz A., Krämer S., Sellge G., Schock M., Bauer M., Schuppan D. (2012) Central role of IL-6 and MMP-1 for cross talk between human intestinal mast cells and human intestinal fibroblasts. Immunobiology 217: 912-917

Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, Boehm G (2002) Dosage-Related Bifidogenic Effects of Galacto- and Fructooligosaccharides in Formula-Fed Term Infants J Pediatr Gastroenterol Nutr 34: 291-295

Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G (2006) A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 91:814–9

Muñoz JA, Chenoll E, Casinos B, Bataller E, Ramón D, Genovés S, Montava R, Ribes JM, Buesa J, Fàbrega J, Rivero M (2011) Novel probiotic *Bifidobacterium longum* subsp. *infantis* CECT 7210 strain active against rotavirus infections. Appl Environ Microbiol 77:8775-8783

Nataro J. (2005) Interactions of the commensal flora with the human gastrointestinal tract. In Nataro J., Cohen P., Mobley H. and Weiser J. Colonization of the mucosa surfaces. American Society for Microbiology Press, Washington, pp 179-186.

Oncel MY, Sari FN, arayci S, Guzoglu N, Erdeve O, Uras N, Oguz SS, Dilmen U (2013) *Lactobacillus reuteri* for the prevention of necrotizing enterocolitis in very low birth weight infants: a randomized controlled trial. Arch Dis Child Fetal Neonatal doi:10.1136/archdischild-2013-304745

Osborn DA, Sinn JKH (2013) Prebiotics in infants for prevention of allergy. Cochrane database Syst Rev Issue 3. DOI: 10.1002/14651858.CD006474.pub3.

Osterballe M, Hansen TK, Mortz CG, Høst A, Bindslev-Jensen C (2005) The prevalence of food hypersensitivity in an unselected population of children and adults. Pediatr Allergy Immunol 16:567–73

Ouwehand, A.C., Isolauri, E., He, F., Hashimoto, H., Benno, Y., Salminen, S (2001) Differences in *Bifidobacterium* flora composition in allergic and healthy infants. J Allergy Clin Immunol 108:144-145

Owen CG, Martin RM, Whincup PH, Smith GD, Cook DG (2005) The effect of infant feeding on the risk of obesity across the life course: a quantitative review of published evidence. Pediatr 115:1367-1377

Özdemir Ö (2009) Gut flora development in infancy and its effect on immune system. Çocuk Enf Derg J Pediatr Inf 3:202-203

Palma GD, Capilla A, Nova E, Castillejo G, Varea V, Pozo T, Garrote JA, Polanco I, López A, Ribes-Koninckx C, Marcos A, García-Novo MD, Calvo C, Ortigosa L, Peña-Quintana L, Palau F, Sanz Y (2012) Influence of milk-feeding type and genetic risk of developing coeliac disease on intestinal microbiota of infants: the PROFICEL study. PLoS One 7:e30791

Parche S., Beleut M., Rezzonico E., Arigoni F., Titgemeyer F., Parche S., Beleut M. (2006) Lactose-over-Glucose Preference in *Bifidobacterium longum* NCC2705: glcP , Encoding a Glucose Transporter , Is Subject to Lactose Repression. J Bacteriol 188:1260-1265.

Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, Adams H, van Ree R, Stobberingh EE (2006) Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut 56:661-667

Pfenninger A, Karas M, Finke B, Stahl B (2002) Structural analysis of underivatized neutral human milk oligosaccharides in the negative ion mode by nano-electrospray MS(n) (part 1: methodology). J Am Soc Mass Spectrom 13:1331–40

Prescott SL, Smith P, Tang M, Palmer DJ, Sinn J, Huntley SJ, Cormack B, Heine RG, Gibson RA, Makrides M (2008) The importance of early complementary feeding in the development of oral tolerance: concerns and controversies. Pediatr Allergy Immunol 19:375–80

Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6:306-314

Roberfroid M (2007) Prebiotics: the concept revisited. J Nutr 137:830S–7S.

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco M-J, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A (2010) Prebiotic effects: metabolic and health benefits. Br J Nutr 104 Suppl :S1–63.

Salminen S, Isolauri E, Endo A, Scalabrin D (2014) Early gut colonization with lactobacilli in vaginally born infants fed breast milk or infant formula (637.9). FASEB J 28:637-639

Satokari R, Vaughan E, Favier F, Dore J, Edwards C, de Vos W (2002) Diversity of *Bifidobacterium* and *Lactobacillus* spp. in breast-fed and formula-fed infants as assessed by 16S rDNA sequence differences. Microb Ecol Health Dis 14:97–105

Saulnier DMA, Spinler JK, Gibson GR, Versalovic J (2009) Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Curr Opin Biotechnol 20:135-41

Savino F, Cordisco L, Tarasco V, Calabrese R, Palumeri E, Matteuzzi D (2009) Molecular identification of coliform bacteria from colicky breastfed infants. Acta Paediatr 98:1582-1588

Savino F, Cordisco L, Tarasco V, Locatelli E, Di Gioia D, Oggero R, Matteuzzi D (2011) Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC microbial 11:157

Savino F, Cordisco L, Tarasco V, Palumeri E, Calabrese R, Oggero R, Roos S, Matteuzzi D (2010) *Lactobacillus reuteri* DSM 17939 in infantile colic: a randomized, double-blind, placebo-controlled trial. Pediatr 126:526-533

Savino F, Pelle E, Palumeri E, Oggero R, Miniero R (2007) *Lactobacillus reuteri* (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. Pediatr 119:124-130

Schouten B, Van Esch BCAM, Kormelink TG, Moro GE, Arslanoglu S, Boehm G, Knippels LMJ, Redegeld FA, Willemsen LEM, Garssen J (2011) Non-digestible oligosaccharides reduce immunoglobulin free light-chain concentrations in infants at risk for allergy. Pediatr Allergy Immunol 22:537–42

Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, Hardt PD (2010) Microbiota and SCFA in lean and overweight healthy subjects. Obesity 18:190-195

Soh SE, Aw M, Gerez I, Chong YS, Rauff M, Ng YPM, Wong HB, Pai N, Lee BW, Shek LPC (2009) Probiotic supplementation in the first 6 months of life in at risk Asian infants – effects on eczema and atopic sensitization at the age of 1 year. Clin Exp Allergy 39:571-578

Solís G, de Los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M (2010) Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe 16:307-310

Stockman JA (2009) Newborn: probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials. In: Deshpande G, Rao S, Patole S. (eds.) Yearbook of Pediatrics, Philadelphia, pp:441-443

Sudo N, Chida Y, Aiba Y, Sonoda, J, Oyama, N, Yu X, Kubo C, Koga Y (2004) Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J Physiol 558:263-275

Sung V, Heine R, Stock A, Barr R, Wake M (2014) Treating infant colic with the probiotic *Lactobacillus reuteri*: double blind placebo controlled randomized trial BMJ doi: 10.1136/bmj.g2107

Symonds EL, O'Mahony C, Lapthorne S, O'Mahony D, Sharry JM, O'Mahony L, Shanahan F (2012) *Bifidobacterium infantis* 35624 protects against salmonella-induced reductions in digestive enzyme activity in mice by attenuation of the host inflammatory response. Clin Transl Gastroenterol 3:e15

Szajewska H, Gyczuk, Horvath A (2013) Lactobacillus reuteri DSM 17938 for the management of infantile colic in breastfed infant: a ramndomized, double-blind, placebo-controlled trial. J Pediatr 162:257-262

Szajewska H, Ruszczyński M, Radzikowski A (2006) Probiotics in the prevention of antibiotic-associated diarrhea in the children: a meta analysis of randomized controlled trials. J Pedriatr 149:367-372

Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco M-J, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A (2010) Prebiotic effects: metabolic and health benefits. Br J Nutr 104 Suppl :S1–63.

Szilagyi A. (2002) Review article: lactose--a potential prebiotic. Aliment Pharmacol Ther 16: 1591-602

Taibi A, Comelli EM (2014) Practical approaches to probiotics use. Appl Physiol Nutr Metab doi: 10.1139/apnm-2013-0490

Tennyson CA, Friedman G (2008) Microecology, obesity, and probiotics. Curr Opin Endocrinol Diabetes Obes 15:422-427

Tham CSC, Peh KK, Bhat R, Liong MT (2011) Probiotic properties of bifidobacteria and lactobacilli isolated from local dairy products. Ann Microbiol 62:1079-1087

Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M (2012) Diversity of bifidobacteria within the infant gut microbiota. PLoS One 7:e36957

Underwood MA, Kananurak A, Coursodon CF, Adkins-Reick CK, Chu H, Bennett SH, Wehkamp J, Castillo PA, Leonard BC, Tancredi DJ, Sherman MP, Dvorak B, Bevins CL (2012) *Bifidobacterium bifidum* in a rat model of necrotizing enterocolitis: antimicrobial peptide and protein responses. 71:546-551

Vandenplas Y, De Hert SG (2011) Randomised clinical trial: the synbiotic food supplement Probiotical vs. placebo for acute gastroenteritis in children. Aliment Pharmacol Ther 34:862-867

Verdu EF (2009) Probiotics effects on gastrointestinal function: beyond the gut? Neurogastroenterol Motil 21:477-480

Viljanen M, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M (2005) Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allerg 60:494-500

Wanke M and Szajewska MD (2012) Lack of an effect of lacrobacillus reuteri DSM 17938 in preventing nosocomial diarrhea in childfren: a randomized, double bind, placebo-controlled trial J Pediatr 161:40-43

Ward R. E., Niñonuevo M., Mills, D.a, Lebrilla C.B., German J.B. (2006) *In vitro* fermentation of breast milk oligosaccharides by Bifidobacterium infantis and *Lactobacillus gasseri*. Appl Environ Microbiol 72: 4497-4499

Westerbeek E.M., van den Berg A., Lafeber H.N., Knol J., Fetter W.P.F., van Elburg R.M. (2006) The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 25: 361-368.

Westerbeek EA, van den Berg JP, Lafeber HN, Fetter WP, Boehm G, Twisk JW, van Elburg RM (2010) Neutral and acidic oligosaccharides in preterm infants: a randomized, double-blind, placebo-controlled trial. Am J Clin Nutr 91:679–86

Westerbeek EA, Slump RA, Lafeber HN, Knol J, Georgi G, Fetter WPF, van Elburg RM (2013) The effect of enteral supplementation of specific neutral and acidic oligosaccharides on the faecal microbiota and intestinal microenvironment in preterm infants. Eur J Clin Microbiol Infect Dis 32:269–76

Wiegering V, Kaiser J, Tappe D, Weissbrich B, Morbach H, Girschick HJ (2011) Gastroenteritis in childhood: a retrospective study of 650 hospitalized pediatric patients. Int J Infect Dis 15:401-407

Yin YN, Yu QF, Fu N, Liu XW, Lu FG (2010) Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats. World J Gastroenterol 16:3394-3401

Ziegler E, Vanderhoof JA, Petschow B, Mitmesser SH, Stolz SI, Harris CL, Berseth CL (2007) Term infants fed formula supplemented with selected blends of prebiotics grow normally and have soft stools similar to those reported for breast-fed infants. J Pediatr Gastroenterol Nutr 44:359–64

PAPER 2

Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants

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MINI-REVIEW

Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants

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Abstract This review is aimed at describing the most recent advances in the gut microbiota composition of newborns and infants with a particular emphasis on bifidobacteria. The newborn gut microbiota is quite unstable, whereas after weaning, it becomes more stable and gets closer to the typical adult microbiota. The newborn and infant gut microbiota composition is impaired in several enteric and non-enteric pathologies. The core of this review is the description of the most recent documented applications of bifidobacteria to newborns and infants for their prevention and treatment. Acute diarrhea is the most studied disease for which bifidobacteria are applied with great success, *Bifidobacterium longum* and *Bifidobacterium breve* being the most applied species. Moreover, the most recent updates in the use of bifidobacteria for the prevention and treatment of pathologies typical of newborns, such as necrotizing enterocolitis, colics, and streptococcal infections, are presented. In addition, a number of not strictly enteric pathologies have in recent years evidenced a strict correlation with an aberrant gut microbiota in infants, in particular showing a reduced level of bifidobacteria. These diseases represent new potential opportunities for probiotic applications. Among them, allergic diseases, celiac disease, obesity, and neurologic diseases are described in this review. The preliminary use of bifidobacteria in in vitro systems and animal models is summarized as well as preliminary in vivo studies. Only after validation of the results via human clinical trials will the potentiality of bifidobacteria in the prevention and cure of these pathologies be definitely assessed.

Keywords Bifidobacteria · Gut microbiota · Infants · Newborns · Probiotics · Therapeutic microbiology

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Introduction

The microbial composition of the gastrointestinal tract (GIT) in humans changes during life. Three different phases can be described almost in all individuals and they correspond respectively to birth, when the microbiota starts to colonize the sterile bowel, to the weaning period, when a different diet determines profound changes giving rise to a stable community that will be present up to the elderly period, when further strong changes in the microbiota occur.

The intent of this review is to focus on the microbiota of infants from birth to the weaning period with a particular emphasis on bifidobacteria. The most recent documented applications of bifidobacteria to newborns and infants for the prevention and treatment of the most common enteric and non-enteric pathologies are described. Moreover, possible applications of these probiotic bacteria for the treatment of diseases for which the probiotic approach appears promising on the basis of recent in vitro studies are suggested. Therefore, this paper represents a comprehensive review of the role of indigenous and administered *Bifidobacterium* spp. in newborns and infants through the description of results collected in the authors' laboratory and obtained by other distinguished scientists. The application of probiotic bacteria such as bifidobacteria for the prevention and therapy of diseases is an emerging sector of applied microbiology which has been referred to as "therapeutic microbiology."

Microbiota composition in newborns and factors affecting microbial colonization

The bowel of neonates resembles that of a germ-free animal. Microbial colonization begins soon after birth and within a few hours bacteria start to appear in the feces (Hansen et al. 2012). Initially, microorganisms are acquired by the contact

with the mother; successively, the newborn is exposed to microorganisms from the environment. Table 1 summarizes the most important factors affecting microbial colonization in the gut. The first bacteria encountered in the majority of healthy infants are facultative anaerobes, because of the positive oxidation/reduction potential of the neonates' intestine at birth. These bacteria remain predominant during the first few days of life, among them, *Staphylococcus* spp., *Enterobacteriaceae*, and *Streptococcus* spp. are most commonly isolated from newborn feces after birth. Facultative anaerobic bacteria are followed by strict anaerobes such as members of the *Bifidobacterium*, *Bacteroides*, and *Clostridium* genera.

The hospital environment is extremely important for intestinal colonization of infants born by cesarean section. These newborns do not come in contact with the maternal vaginal and fecal microorganisms and may be separated from the mother for a long period after birth (Biasucci et al. 2010). Within the largest epidemiologic study performed on newborns and focused on the determination of factors affecting gut composition (the KOALA study, Penders et al. 2006), it has been demonstrated that anaerobic colonization, especially by *Bacteroides* spp., is delayed in cesarean section newborns, but *Bifidobacterium* spp. retrieval and *Escherichia coli* presence were similar in vaginally and cesarean section-delivered infants. Additionally, an increased incidence of *Clostridium perfringens* and *Clostridium difficile* in cesarean section newborns is reported in relation to the hospital environment. Another important factor that can influence the composition of the intestinal microbiota in neonates is the type of feeding (Table 1) as revealed by the KOALA study. In full-term breastfed neonates, *Bacteroides* spp. and bifidobacteria appear 4 days after birth, and after 1 week, they dominate the fecal microbiota constituting 80–90 % of the total microbial amount. In contrast, the fecal microbiota of formula-fed infants is more complex, with *Bifidobacterium* spp., enterobacteria, and *Streptococcus* spp. in similar proportion. Another notable difference is that formula-fed infants have much higher counts of *Clostridium* spp. than breastfed infants. An important difference is the relative buffering capacity of the two feeds. Breast milk has a poor buffering capacity, compared with formula milk, and this leads to marked differences in the colon pH of breast and formula-fed infants—5.1 and 6.5, respectively. The low pH promotes the growth of bifidobacteria and lactobacilli, but is inhibitory to many other bacteria (Tham et al. 2011). Moreover, a number of peptides capable of stimulating the growth of several bifidobacteria have recently been isolated from human milk. In addition, human milk contains glycoproteins, glycolipids, fucose, neuraminic acid, lactose, *N*-acetylglucosamine, and a variety of oligosaccharides that are known to possess a bifidogenic effect (Coppa and Gabrielli 2008).

Table 1 Principal factors influencing intestinal microbiota development in newborns

Place and mode of delivery
Maternal microbiota of intestine, vagina, and epidermis
Type of infant feeding (breast milk vs. formula milk)
Antibiotic/antimycotic use in newborns
Gestational age at birth
Hospitalization after birth
Perinatal administration of probiotics
Intrapartum antibiotic prophylaxis?

Both neonates and adults are regularly exposed to microorganisms via the diet, but with different effects: microorganisms entering newborns are more likely to colonize than those entering healthy adults possessing a stable microbiota. Breast milk is a potential source of microorganisms, although the results available to date about the isolation and identification of commensal or potential probiotic bacteria from milk of healthy women are still inconclusive. Even though authors are aware that human milk is difficult to sample and microbial contamination can never be totally discarded, some studies have demonstrated the presence of live bifidobacteria in human milk (Solís et al. 2010; Arboleya et al. 2011).

In contrast with full-term neonates, little information concerning the composition of the microbiota in premature infants is available because the interindividual variability is higher than in full-term newborns and many parameters, such as antibiotic treatments and diet, may tend to increase study discrepancy. In particular, preterm infants often need parental feeding and respiratory support, and they are vulnerable for infections and often require antibiotic treatment. Moreover, gastric pH of preterm infants is higher than that of term infants probably due to more frequent feeding and it leads to a greater risk of bacterial infections. The alteration in the composition of the gut microbiota of preterm infants can be linked to the increased risk, for this subjects, of severe gastrointestinal disorders such as necrotizing enterocolitis (NEC) (Lin et al. 2008; Barrett et al. 2013).

Microbiota composition after weaning

The primary factor involved in the compositional shift in the microbiota is the change of diet (Bäckhed et al. 2004; Koenig et al. 2011). With the introduction of solid foods (weaning), at about 6 months of life, the infants are exposed to more complex carbohydrates and other nutrients with respect to those present in human milk or infant formula, and these new substrates drive to the development an adult-like microbiota. In this context, a high gene expression for more complex carbohydrates degradation by representative microbial groups is observed as well (Koenig et al. 2011).

A large-scale longitudinal study on development and change in the composition of gut microbiota during the

process of weaning was carried out by Fallani et al. (2011). Within this study, fecal samples of 605 infants (from five European countries), approximately 4 and 6 weeks after the introduction of first solid foods, were collected in order to investigate the association with determining factors such as mode of delivery, previous feeding practices, age of weaning, and the impact of possible antibiotic treatment. After 1 month of weaning, bifidobacteria and *Bacteroides* continue to represent the predominant groups. However, the number of detectable *Bifidobacterium* species decreased after weaning together with counts of *C. perfringens* and *C. difficile*, while other strictly anaerobic clostridia increased. Furthermore, the same authors also pointed out how, after 6 months of weaning, the influence of feeding method and the mode of delivery persisted. High counts of bifidobacteria are associated with breastfeeding until and during weaning; in addition, the delay and the consequent low anaerobic colonization, especially by *Bacteroides* spp., in cesarean section newborns was still present in this period (Fallani et al. 2010; Biasucci et al. 2010). On the contrary, it has been reported that the effect of antibiotic treatment in infants or their mother on gut microbiota composition disappeared after weaning started (Fallani et al. 2011). Fecal microbiota at weaning due to effects of the dietary changes were analyzed using PCR-temporal temperature gradient gel electrophoresis (TTGE) of DNA isolated from infants feces. This technique evidenced a high interindividual variability in the dominant microbiota, which slightly decreased after cessation of breastfeeding and the introduction of solid foods. In the TGGE profiles, the main identified bands present from the breastfeeding period to the post-weaning time corresponded to *E. coli*, *Ruminoccocus* spp., and bifidobacteria (Magne et al. 2006). Therefore, the gut microbiota of infants, due to change of diet, becomes more stable and homogenous after weaning and it gradually gets closer to the typical adult microbiota (Magne et al. 2006; Koenig et al. 2011).

Bifidobacterial biodiversity in healthy newborns and infants

Bifidobacteria were first characterized from infant feces by Tissier at the very beginning of the twentieth century with the isolation of a bacterium with a peculiar Y shape which was named *Bacillus bifidus*. Only about 50 years later, with the discovery of a peculiar metabolic pathway for hexose fermentation in Y-shaped bacteria, the *Bifidobacterium* genus was defined and separated from the *Lactobacillus* genus. Historical details of the *Bifidobacterium* genus are described in Biavati et al. (2000).

The distribution of the *Bifidobacterium* species in the feces of newborns was originally obtained with traditional plate isolation technique. Biavati et al. (1984) studied the microbial composition of breastfed and formula-fed newborns by culture methods and DNA–DNA hybridization as identification tool. It was found that the most represented species in both groups of infants were *Bifidobacterium infantis* (at present classified as *B. longum* subsp. *infantis*), *Bifidobacterium breve*, *Bifidobacterium longum* (at present classified as *B. longum* subsp. *longum*), and *Bifidobacterium bifidum*. *Bifidobacterium pseudocatenulatum* and *Bifidobacterium catenulatum* were also present although in a lower number in both type of samples, whereas *Bifidobacterium dentium* was evidenced only in breastfed newborns.

A technical difficulty encountered in microbial ecology studies performed before the advent of the molecular techniques was that not all components of the GIT microbiota are cultivable (Favier et al. 2002). In the last 20 years, analysis methods based on the detection and sequencing of 16S rDNA have been widely used in place of conventional culture methods. PCR-denaturing gradient gel electrophoresis (DGGE) with the use of universal bacterial primers was used for monitoring bifidobacterial succession in the feces of a breastfed and a mixed-fed newborn (an increased amount of formula milk was added to the diet starting from 2 weeks) (Favier et al. 2002). Both newborns showed an early colonization by bifidobacterial species, which were detected starting from the third day of life. Among them, *B. breve* was the most represented species. Differences in the bifidobacterial pattern were appreciated when the newborn feeding started to change: amplicons related to bifidobacteria were dominant in breastfed babies during the first 6 months of life, whereas in the babies who had a mixed feeding, these amplicons were less intense. The introduction of solid food and the withdrawal of breast milk resulted in major shifts in the bifidobacterial profiles. TGGE studies of DNA amplified from infant feces collected during the breastfeeding period and the successive weaning period evidenced that bifidobacteria were the predominant group, precisely *B. infantis*, *B. longum*, and *B. breve*, and that in the post-weaning period, bifidobacteria bands tend to diminish of intensity indicating a decrease in the *Bifidobacterium* population (Magne et al. 2006).

Important information on the temporal development of bifidobacteria in newborns was obtained by Klaassens et al. (2009) with a qPCR approach. Their analyses confirmed what traditional plate counts had already stated, i.e., that total bifidobacteria number was higher in breastfed newborns than in formula-fed ones, but the number in formula-fed newborns increased significantly over time, due to the intake of the oligosaccharides present in the formula. qPCR analyses of targeted *Bifidobacterium* species indicated that there were few significant differences between the breastfed and formula-fed newborns and that the number of these species in breastfed newborn was initially higher. *Bifidobacterium animalis* and *B. dentium* were not detectable in samples from all infants, whereas *B. longum* subsp. *infantis*, *B. breve*, *B. bifidum*, and *B. longum* subsp. *longum* were detected in all

samples, with *B. longum* subsp. *infantis* being the major species found. *Bifidobacterium adolescentis*, which is most commonly found in adults, was present only in formula-fed infants. The same authors also determined the bifidobacterial transcriptome with a DNA microarray based on 6,000 clones from a library derived from a mixture of six *Bifidobacterium* species. The results evidenced a significant impact of the diet (breast- and formula-feeding) on the transcriptional response of bifidobacteria. The expression of the glycobiome, in particular the genes encoding for pullulanases, glucosidases, and the glycogen phosporylase, indicates a higher potential for carbohydrate metabolism in breastfed newborns than in formula-fed ones (Klaassens et al. 2009).

In contrast to the abovementioned studies, some metagenomic analyses revealed a low abundance of bifidobacteria (Palmer et al. 2007; Koenig et al. 2011). Turroni et al. (2012) delineated that the low counts obtained by these investigations were most likely due to technical biases, in particular related to DNA extraction protocols and/or the PCR primers used. The same authors (Turroni et al. 2012) gave further insight in the complexity and biodiversity of bifidobacteria in healthy newborns by pyrosequencing of PCR amplicons derived from two hypervariable regions of the 16S rRNA gene. This study finally elucidated that the most abundant class in infant fecal samples was *Bifidobacteriales*, being present at 80.6 %. The predominant bifidobacterial species detected were *B. longum* and *B. bifidum* at 56.2 and 10.7 %, respectively. Regarding the interindividual variability in the infant gut microbiota, the statistical analyses performed revealed a large conservation of members of the *Actinobacteria* with a high proportion (ranging from 21.7 to 90.6 %) belonging to the *Bifidobacteriaceae* family. In particular, the *B. breve* species was always detected with an average of 5.5 % of total reads. In contrast, *B. adolescentis* was detected in a relatively high average percentage (3.4 %), but it was only present in about 2 % of the subjects. Notably, 3.7 % of the total number of reads was assigned to uncultured bifidobacterial phylotypes retrieved from human fecal samples and 0.23 % of bifidobacterial sequences had not been identified previously. This study also outlined the power that next generation sequencing technology might have in a clear definition of the infant gut microbiota and in the understanding of the parameters that influence colonization, development, and composition of the microbiota from an early stage following birth, and to define its beneficial activities into subsequent life stages.

Bifidobacterium spp. as probiotic strains in newborns and infants

Bifidobacteria are widely used as probiotics for preventive and therapeutic purposes in newborns and infants considering their high abundance in the GIT tract, their capability of colonizing the gut, and their long history of safe use (Sanders et al. 2010). The concept of beneficial bacteria was originally proposed in 1906 by Tissier, who promoted the administration of what he called *Bacillus bifidus* to infants with diarrhea, basing on the concept that the beneficial bacteria will replace those responsible for the intestinal disturbance (Kailasapathy 2008). A large number of studies and application of *Bifidobacterium* spp. to newborns and children are present in the literature, but only in 2002 the Food and Drug Administration has given to *Bifidobacterium lactis* the "generally regarded as safe (GRAS)" status (Hammerman et al. 2006) and authorized its use in formula milks. The positive effects on the administration of *B. lactis* Bb12 on the reestablishment of a balanced composition of the gut microbiota were found on preterm, full-term newborns, and toddlers (Mohan et al. 2006). Other *Bifidobacterium* species have successively received the GRAS status and have entered the list of strains possessing the quality and presumption of safety (QPS) status by the European Food Safety Authority. A comparison of the two safety assessment systems is given in Wassenaar and Klein (2008). Among the different species belonging to this genus, *B. breve* appears to be one of the most used in infants. It has been assessed that the very early administration (at the first days of life) of a *B. breve* strain to low birth weight infants was useful in promoting the colonization of the bifidobacteria and the formation of a normal intestinal microbiota (Li et al. 2004).

The administration of probiotics to newborns and infants is described in this review, starting from the most common application for the prevention and treatment of acute diarrhea (Table 2) to most recent approaches. The description of these innovative applications has been divided into two sections, the first one focused on newborn pathologies (Table 3) (necrotizing enterocolitis, infantile colics, and group B streptococcal neonatal infection) and the second one on infant diseases (Table 4) (allergy, celiac disease, obesity, neurological and psychiatric diseases). A number of these pathologies are not strictly gut dysfunctions, but some of them have in recent years been correlated to alterations of the gut microbiota, in particular with a reduced level of bifidobacteria. These diseases represent new potential opportunities for probiotic application.

Bifidobacterium spp. strains for the treatment and prevention of acute diarrhea in newborns and infants

One of the best-studied clinical outcomes with the use of probiotic bacteria is acute diarrheal disease. Infectious gastroenteritis is one of the leading causes of morbidity especially in newborns and children under 5 years of age (Wiegering et al. 2011). Although gastroenteritis-associated mortality is rare in Western Europe, an increased incidence has been noted in some national registers over recent years. The most common

Table 2 Overview of bifidobacteria applications as probiotics for the treatment of acute diarrhea

Pathology	Probiotic microorganism	Reported effect(s)	References
Acute diarrhea	*B. animalis* subsp. *lactis* BB-12	Increased immune mechanisms	Weizman et al. (2005)
	B. animalis subsp. *lactis* strain	Decreased duration of diarrhea	Grandy et al. (2010)
	B. animalis subsp. *lactis* plus *B. longum* subsp. *infantis* strains	Decreased duration of diarrhea	Vandenplas and De Hert (2011)
	B. breve DSM 24706, *B. breve* DSM 24707, *B. breve* DSM 24708, *B. longum* subsp. *longum* DSM 24709	Increased inhibition of diarrhea pathogens (in vitro study)	Aloisio et al. (2012)
	B. breve Yakult	Decreased incidence of diarrhea	Wada et al. (2010)

causes of infant gastroenteritis are viruses and bacteria, but parasite infections are also diffused. Bacterial infections are more evident in the early months of infancy, whereas from 6 months to 2 years of age, rotavirus is the most common etiologic agent worldwide, followed by adenovirus and norovirus (Wiegering et al. 2011). Bacterial and viral gastroenteritis present with different clinical features. Rotavirus infections are known to be more severe and more often associated with a complicated course. In the last few decades, several bacteria (e.g., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *C. difficile*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *E. coli*) and parasites (e.g., *Cryptosporidium* spp.) have been identified as important causes of diarrhea in humans, particularly in infants (Amisano et al. 2011). Diarrheagenic *E. coli* represents one of the most bacterial causes of pediatric diarrhea in developing countries. *E. coli* is usually found in the commensal intestinal microbiota, but it can become a pathogen through acquisition of genetic determinants, which may enhance adhesiveness and toxicity. *C. difficile*, *K. pneumonia*, and *E. clocae* are also commensals but they can cause secondary bacteremia in the relatively vulnerable intestinal wall of young infants especially after mucosal damage due to rotavirus infection (Lowenthal et al. 2006).

The effect of different probiotic species and strains on the recovery from acute diarrhea is currently well accepted. The majority of the studies have included various species of bifidobacteria (Table 2) and lactobacilli, and, by far, the most used have been *B. animalis* subsp. *lactis*, *Lactobacillus rhamnosus* LGG, and *Lactobacillus reuteri* (Weizman et al. 2005; Indrio and Neu 2011). The largest number of trials documents the therapeutic use of probiotics as supplements early in the course of the disease, the most consistent effect being a reduction in duration of illness. Using a different approach, other authors have examined a preventive administration of probiotics. These studies documented a reduction in incidence or severity of acute diarrheal disease (Saavedra and Tschernia 2002). Moreover, several probiotic strains resulted effective in reducing the risk of antibiotic-associated diarrhea in newborns and children. A clinical trial, performed with 766 infants, indicated that treatment with a probiotics strain (LGG), compared with placebo, reduced the risk of diarrhea from 28.5 to 11.9 % (Szajewska et al. 2006).

A recent study has been focused on the treatment of diarrhea caused by rotavirus using bifidobacteria (Muñoz et al. 2011). In particular, in this study a *B. longum* susp. *infantis* strain, isolated from infant feces, was first selected for the capability of inhibiting in vitro rotavirus replication and its capability to protect cells from virus infection and then it was tested for the in vivo treatment on a mouse model. The results demonstrated the efficacy of this *Bifidobacterium* strain against rotavirus infection. Clinical trials in children hospitalized for acute rotavirus diarrhea confirmed the efficacy of *Bifidobacterium* strains belonging to the *longum* and *animalis* species in combination with other probiotic strains for the treatment of the disease. Mixtures of probiotics showed good results in the reduction of duration and the severity of the disease (Grandy et al. 2010; Vandenplas and De Hert 2011). In addition, supplementation of bifidobacteria to hospitalized

Table 3 Overview of current and potential applications of bifidobacteria in newborns

Pathology	Probiotic microorganism	Reported effect(s)	References
Necrotizing enterocolitis (NEC)	*B. breve* M16-V	Reduced production of butyric acid	Wang et al. (2007)
	B. bifidum strain in Infloran product	Reduced incidence of NEC and death	Lin et al. (2008)
	B. breve strain (Yakult preparation)	Increased intestinal motility	Braga et al. (2011)
Infantile colics	*B. breve* DSMZ24706, *B. breve* DSMZ24707, *B. breve* DSMZ24708, *B. longum* DSMZ24709	Antimicrobial activity against gas-forming coliforms (in vitro study); clinical trial is ongoing	Aloisio et al. (2012)
Group B streptococcal neonatal infection	No studies available yet		

Table 4 Overview of current and potential applications of bifidobacteria in infants

Pathology	Probiotic microorganism	Reported effect(s)	References
Allergies	*B. animalis* subsp. *lactis* BB-12	Decreased severity of atopic eczema	Isolauri et al. (2000)
	B. breve BB99	Reduced eczema caused by cow's milk allergy	Viljanen et al. (2005)
	B. longum BL999	No effect on prevention of eczema	Soh et al. (2009)
	B. breve BB99	Decreased incidence of IgE-associated allergy	Kuitunen et al. (2009)
Celiac disease	*Bifidobacterium* spp. strains	Positive effects on inflammatory and allergic bowel disease (human cell lines)	Young et al. (2004)
	B. longum susp. *longum*, *B. breve*, *B. longum* subsp. *infantis* in VLS#3 product	Decreased toxicity of gluten (murine model and human cell lines)	De Angelis et al. (2006)
	B. animalis subsp. *lactis*	Reduced damage induced by gliadin (human cell lines)	Lindfors et al. (2008)
Obesity	*B. longum* strain	Decreased serum total cholesterol (murine model)	Xiao et al. (2003)
	Bifidobacterium spp. strains	Increased glucose tolerance and decreased proinflammatory cytokines (murine model)	Cani et al. (2007)
	B. longum susp. *longum*, *B. breve*, *B. longum* susp. *infantis* in VLS#3 product	Increased hepatic natural killer T-cell (murine model)	Ma et al. (2008)
	Bifidobacterium spp. strains	Reduced serum and liver triglyceride (murine model)	Yin et al. (2010)
	B. pseudocatenulatum SPM1204, *B. longum* SPM1205, *B. longum* SPM1207	Reduced serum cholesterol level (murine model)	An et al. (2011)
Neurology and psychiatry	*B. infantis* 35624	Increased level of plasma tryptophan (murine model)	Desbonnet et al. (2008)
	B. longum NCC3001	Decreased infection-induced behavioral changes (murine model)	Bercik et al. (2011)

infants significantly prevented the incidence of diarrhea and the onset of hospital acquired diseases. As an example, the beneficial effect of *B. breve* strain Yakult has been evidenced in immunocompromised pediatric patients on chemotherapy. These young patients suffered from infectious complications; following probiotic administration, the use of antibiotics to cure infections was lower and the gut habitation of anaerobes was enhanced (Wada et al. 2010). Several *Bifidobacterium* strains, deriving from infant feces and mainly belonging to the *B. breve* species, were capable of contrasting the growth of pathogens causing infectious diarrhea of bacterial origins in infants, making them potential probiotic candidates for a formulation aimed at the prevention or the cure of bacterial diarrhea (Aloisio et al. 2012). Furthermore, a recent study demonstrated the efficacy of *B. longum* subsp. *infantis* 35624 of reducing the effect of villi-associated enzyme caused by *Salmonella* infection. The mechanism of the strain could be linked to the modulation of the immune response of the host (Symonds et al. 2012).

Bifidobacterium spp. strains for the treatment and prevention of pathologies in newborns

Necrotizing enterocolitis (NEC)

Despite advances in neonatal care, NEC still remains the leading cause of morbidity and mortality in neonatal intensive care units (Hunter et al. 2008). NEC has a multifactorial etiology leading to inflammation and necrosis of the neonatal intestine. Gastrointestinal dysfunction can progress to pneumatosis intestinalis, systemic shock, and rapid death in severe cases (Neu and Walker 2011). Several epidemiologic studies have identified multiple factors that increase infant's risk for the development of NEC, such as prematurity, enteral feeding, bacterial colonization, or a synergy of these three factors (Claud and Walker 2001). There is a strong evidence that the initial bacterial colonization after birth plays a pivotal role in the development of NEC. Colonization by commensal bacteria is required for the normal development and maturation of the newborn intestine. Preterm newborns, who are at increased risk of developing NEC, show a different colonization with respect to full-term newborns. In preterm neonates, facultative anaerobes, such as enterobacteria and enterococci, some of which are potentially pathogenic bacteria, remain predominant until the 20th day of life. For this reason, it has been suggested that a major etiological factor for NEC is the abnormal microbiota, particularly as NEC usually occurs after 8–9 days postpartum when usually anaerobic bacteria are colonizing the gut (Mai et al. 2011). It is also true that premature newborns have an immature and inappropriate intestinal epithelial immunologic response to luminal bacterial stimuli (Claud and Walker 2001). Several studies have shown that formula-fed infants have a higher incidence of NEC than breastfed infants, and this is due to the fact that breast milk contains passive immunity factors such as polymeric IgA and

has a bifidogenic effect that enhances intestinal maturation and provides protection to the newborn (Sisk et al. 2007; Sullivan et al. 2010).

Many studies have shown the efficacy of probiotics for prevention and reduction of incidence of NEC although differences were observed between the types of microorganisms, the dosage, and the time of use. *Bifidobacterium* spp. are widely used for this purpose (Table 3). Wang et al. (2007) evidenced that the administration of a *B. breve* strain to low birth weight infants reduced the production of butyric acid, which may be helpful in protecting these infants from NEC. Several authors have reported systematic reviews of randomized and controlled trials of probiotic supplementation in preterm infants (Deshpande et al. 2007; Barclay et al. 2007; Stockman 2009; Deshpande et al. 2010). The results of clinical trials reported that the incidence of NEC was reduced in neonates receiving probiotic supplementation compared with control groups. The action attributed to probiotics is species specific (Verdu 2009). Administration of *B. bifidum*, *B. breve*, and *B. animalis* susp. *lactis* strains in preterms and low birth weight infants showed evident clinical benefits for the treatment of NEC (Lin et al. 2008; Khailova et al. 2009; Braga et al. 2011; Underwood et al. 2012). The principal effects that can explain the efficacy of bifidobacteria are resistance to colonization of potential pathogenic microorganism, immunomodulation, nutritional contribution, and improved intestinal motility. Treatment with *B. breve* associated with *L. casei* in 231 preterm infants has underlined a positive correlation between improved intestinal motility and NEC (Braga et al. 2011). Khailova et al. (2009) and Underwood et al. (2012) investigated the effect of a *B. bifidum* OLB6378 strain on a rat model and an intestinal epithelial cell line (IEC-6). Administration of *B. bifidum* OLB6378 increased expression of some genes involved in mechanisms of protection against mucosal infection such as antimicrobial peptides and apoptosis regulation. Because of the complexity of the pathogenesis of NEC, the administration of different *Bifidobacterium* strains may benefit by different actions on the host, and therefore, multistrain probiotic preparations are likely more effective than single-strain ones (Deshpande et al. 2011).

Infantile colics

A new aspect of the application of bifidobacteria and probiotics in general is the treatment of gas colics in newborns. Infantile colics are a common condition in the first months of life, and about 10–30 % of infants are affected by this disorder. Infants affected by colics suffer from paroxysms of excessive, inconsolable crying, frequently accompanied by flushing of the face, meteorism, drawing-up of the legs, and passing of gas. Although infantile colic is a common disturbance, the etiology remains obscure; however, evidences suggest multiple independent causes.

An abnormal intestinal microbiota has been proposed to affect gut function and gas production that lead to colicky behavior. Gas-forming coliforms were in fact found to be more abundant in colicky newborns with respect to noncolicky ones (Savino et al. 2009). A comparison between the microbiota of colicky and noncolicky newborns performed using a phylogenetic microarray has pointed out the presence of peculiar microbial signatures, in particular high abundance of proteobacteria including gas-forming bacteria and low abundance of Bacteroidetes and Firmicutes, in the first week of life in neonates who develop colics. These microbial signatures may be used for early diagnostics as well as for developing specific therapies (de Weerth et al. 2013).

Regarding possible therapies, the study of Savino et al. (2007) examined, for the first time, the modulation of intestinal microbiota of colicky infants by administering a probiotic strain. A cohort of 90 breastfed colicky infants was randomly assigned to treatment with a strain of *Lactobacillus reuteri* (ATCC 55730) and simethicone. This study evidenced that infants treated with *L. reuteri* had a significant reduction in crying compared to infants treated with simethicone. The positive effect of probiotic administration on the reduction of gas colic symptoms and on the modulation of intestinal microbiota was confirmed in a successive trial with the strain *L. reuteri* DSM 17938 (Savino et al. 2010). These studies have given a new input on the use of probiotics for the treatment of colics and have stimulated the research of new probiotic strains (Savino et al. 2011; Aloisio et al. 2012). Recently, a selection of *Bifidobacterium* strains to be used on newborns for the treatment of enteric disorders with a special attention on colics was carried out (Aloisio et al. 2012). The strains were selected for their capability of inhibiting the growth of pathogens typical of the newborn GIT, including gas-forming coliforms. Finally, the large array of aspects examined in this study, including safety properties according to the EFSA guidelines, has allowed the identification of three *B. breve* strains and one *B. longum* subsp. *longum* strain as potential probiotics for the treatment of infantile colics in newborn (Table 3). A clinical trial aimed at the in vivo validation of the effect against colics of some of the selected strains has just started but results are not available yet (authors' personal communication).

Group B streptococcal neonatal infection

Early-onset bacterial sepsis remains one of the major causes of neonatal morbidity and mortality although the sepsis-associated death rates have declined significantly in the last decade (2001–2011) (Ferrieri and Wallen 2012). The reason of the reduction of mortality is due to the introduction of an intrapartum antibiotic prophylaxis, during labor and delivery, in group B *Streptococcus* (GBS)-positive pregnant women (Puopolo et al. 2005). The leading cause of onset infection of

fetus and newborn is GBS. This gram-positive bacterium, resides in the cervix, vagina, or rectum, can reach the amniotic through intact or rupted membranes and lead to infection. Identification of maternal colonization by GBS during pregnancy is very important for taking preventive measures, such as antibiotic prophylaxis, against neonatal disease. GBS can cause two types of infections in newborns, the early-onset and late-onset infections. They are very different: in the first case, it manifests with respiratory disturbance and apneic episodes, while in the second case with fever and poor feeding. Whereas the introduction of antibiotic maternal prophylaxis has significantly decreased the incidence of GBS for the early-onset disease, there is no evidence that chemoprophylaxis prevents late-onset disease. Moreover, there are only a few information in the literature about the effect that the antibiotic treatment may have on the early colonization of bacteria in the newborn gut (Corvaglia et al. 2012), which is known to be highly influenced by the microorganisms that are derived from the mother (Table 1). The possibility of using probiotics as anti-streptococcal agents in pregnant woman represents a possible way of reducing GBS infections in newborns without any alteration of the gut microbiota (Table 3). In our laboratory, studies are currently been performed aimed at the evaluation of the antimicrobial activity against *Streptococcus agalactiae* of several *Bifidobacterium* strains which can have a positive effect both on the mother and on the newborn (unpublished results).

Bifidobacterium spp. strains for the treatment and prevention of pathologies in infants

Allergies

Industrialized countries successfully controlled infectious diseases during the second half of the last century, by improving sanitation and using antibiotics and vaccines. Therefore, the incidence of infectious disease deriving from poor hygienic conditions is declining (EFSA 2013). At the same time, the incidence of allergic disorders such as atopic dermatitis, rhinitis, and asthma has increased strikingly in developed countries. One of the most reliable hypotheses of this increase is a relative lack of microbial stimulation and a failure of immunoregulation due to low exposure of the infantile gut immune system to harmless microorganisms associated with the environment (Cabana et al. 2007). This hypothesis is supported by the clear evidence that immunoregulation is faulty in individuals suffering from allergies (Isolauri 2004). Furthermore, epidemiological data have shown that atopic children have a different intestinal microbiota from that of healthy children, with higher levels of clostridia and lower levels of bifidobacteria. In addition to the lower number of bifidobacteria, infants suffering from atopic disease harbor a peculiar pattern of bifidobacteria comprising adult-like strains, such as *B. adolescentis*, as compared to healthy infants with a typical infant pattern (Ouwehand et al. 2001). Considering that the adult-like microbiota resembles that of formula-fed infants, it has been suggested that the bifidogenic factors present in breast milk favor the development of infant-type microbiota which may in turn protect from the development of atopic disease. The *Bifidobacterium* species of allergic infants also have a reduced adhesion to human intestinal mucus, a phenomenon which is likely to alter host–microbe interactions during the first months of life (Ouwehand et al. 2001). In addition, *Bifidobacterium* spp. from allergic infants induce less IL-10 and more proinflammatory cytokine production than those from non-allergic ones (Boyle and Tang 2006). Other studies have also shown that early colonization with pathogenic bacteria is more likely to occur in children who go on to develop allergy; in contrast, lactobacilli and bifidobacteria are found more commonly in the composition of the gut microbiota of non-allergic children and this seems to correlate with protection against atopy (Kalliomaki et al. 2001; Özdemir 2009).

Therefore, the possibility of using probiotics to prevent the development of allergic disease is a feasible option: beyond the probiotic approach is the evidence that the immunophysiological regulation in the gut depends on the establishment of a healthy gut microbiota (Isolauri and Salminen 2008). Several studies suggest that certain probiotic strains exhibit powerful anti-inflammatory capabilities. Specific probiotics, most of them belonging to the *Lactobacillus* or *Bifidobacterium* genera, aid in the regulation of the secretion of inflammatory mediators and in the development of the immune system during the critical period of life when these functions are immature and the risk of allergic disease is increased (Isolauri and Salminen 2008).

A number of studies regard bifidobacteria administration to prevent allergic diseases (Table 4), although the majority of works is focused on lactobacilli administration (Özdemir 2010). A probiotic mixture, containing *B. breve* Bb99 strain in addition to lactobacilli and propionibacteria, was administered to mothers during the last month of pregnancy and their infants received it from birth until age 6 months. The treatment resulted in a decreased incidence of IgE-associated allergy, such as atopic diseases, in cesarean-delivered children until the age of 5, with respect to the administration of a placebo (Kuitunen et al. 2009). The same probiotic mixture was also found to be effective in the treatment of eczema in infants with proven cow's milk allergy (Viljanen et al. 2005). The oral therapy with the probiotic preparation VLS#3 (VSL Pharmaceuticals, Gaithesburg, MD), a mixture of lactic acid bacteria and bifidobacteria, could reduce anaphylactic symptoms in a murine model of allergic sensitization, although no clinical trial has been performed on humans yet (Di Felice et al. 2008).

Conversely, commercially available cow's milk formula supplemented with *B. longum* BL999 and *L. rhamnosus* LPR administered in the first 6 months of life to Asian infants at risk of allergic disease showed no effect on prevention of eczema or allergen sensitization in the first year of life (Soh et al. 2009). The intake of *B. longum* BB536 administered for 13 weeks during the pollen season was found to relieve allergic rhinitis symptoms in adults, but no studies are available on bifidobacteria administration to children (Xiao et al. 2006). The administration of *B. lactis* BB12 applied to a neonatal murine model of asthma was found to suppress all aspects of the asthmatic phenotype (Feleszko et al. 2007), but, also in this case, a clinical trial on humans has not been performed yet. However, in a different study, the same strain added as a supplement to hydrolyzed whey formula was found to significantly reduce the severity of atopic eczema in infants after a 2-month treatment (Isolauri et al. 2000).

Other studies have been focused on the effects of probiotics in the treatment of food allergy, but conclusive effects have not been evidenced yet (Boyle and Tang 2006). Some preliminary positive results have been obtained with LGG supplementation, but no experimentation is available on bifidobacteria.

Celiac disease

Celiac disease (CD) is an autoimmune enteropathy of the small intestinal mucosa induced by the ingestion of wheat gluten, or other similar proteins found in barley and rye, in genetically predisposed individuals (Green and Cellier 2007). CD is characterized by two types of immune response to gluten-derived peptides: an adaptive immune response Th_1-dependent, within the intestinal mucosa, and an innate immune response. Both responses lead to the release of proinflammatory cytokines like IFN-γ and IL-15 (Londei et al. 2005) and result in a consequent inflammation and intestinal tissue remodeling (Meresse et al. 2009).

CD is one of the most common chronic diseases in Europe and USA with a prevalence of about 1–3 % (Mulder and Bartelsman 2005). These percentages are comparable to the prevalence found in a UK pediatric study (Bingley et al. 2004). CD can occur at any age with different clinical forms; however, its classic form usually manifests in early childhood (up to 24 months), after gluten introduction in the diet (Van Heel and West 2006). The classic form of CD is characterized by small bowel mucosal atrophy and malabsorption, and the symptoms include abdominal distention, loss of weight, and chronic diarrhea (Fasano and Catassi 2005). The etiology of CD is complex and regards both genetic and environmental factors (Akobeng et al. 2006; Dubois and Van Heel 2008). As evidenced in several studies, the ingestion of gluten represents a major environmental factor in CD development, but there are also other important factors that play a role in disease risk, such as the type of milk-feeding, the administration of antibiotic, and in particular, the gut microbial composition (Collado et al. 2007). Dietary factors in early childhood play an important role, especially because they influence gut bacterial colonization and its physiological development together with the maturation of the immune system (Palma et al. 2012). Breastfeeding may offer protection against the development of CD (Ivarsson et al. 2002; Akobeng et al. 2006).

As already evidenced, breastfeeding plays an important contribution in shaping the intestinal microbiota (Bezirtzoglou et al. 2011). Several works have shown that the gut microbiota is implicated in immunorelated disorders and that specific probiotic strains, belonging to the genera *Lactobacillus* and *Bifidobacterium*, are able to reduce inflammatory diseases such as allergy and inflammatory bowel disease and normalize gut mucosal dysfunction (Sartor 2004; Gueimonde et al. 2007). Palma et al. (2012) have evaluated the gut microbial colonization during the first 4 months of life in breastfed and formula-fed healthy full-term infants with predisposing genes on CD risk. The authors have demonstrated that the milk-feeding type and the HLA-DQ genes (the major genetic risk factor for CD) influence the gut bacterial colonization. In particular, high levels of the *B. fragilis* group were always found in infants with a genetic CD risk and simultaneously formula-fed but not in those breastfed. In contrast, the reduction of *Bifidobacterium* spp. and in particular *B. longum* counts was associated with an increased CD risk. As reported above, breastfeeding is adept at promoting the colonization of *Bifidobacterium* spp. and then at reducing the microbial gap linked to the HLA-DQ genotype (Palma et al. 2012).

A lower number of gut microbial species has been shown in healthy children than in celiac children by DGGE analyses (Sanz et al. 2007). Conversely, the diversity of *Bifidobacterium* species was significantly higher in healthy children than in celiacs. Other studies have been focused on the characterization of the fecal microbiota composition of celiac children by fluorescent in situ hybridization (FISH) and real-time PCR (Collado et al. 2007, 2008). These studies underlined high levels of *Bacteroides* spp., *Clostridium* spp., and sulfate-reducing bacteria in celiac children and higher *Bifidobacterium* levels in controls with respect to celiac children. According to final evidences, the higher levels of *B. longum* are associated with healthy children, and therefore, this species could exert a protective effect in celiac early childhood (Collado et al. 2008). Nadal et al. (2007) have also found high levels of gram-negative bacteria belonging to the *E. coli* group and proinflammatory species belonging to the *Bacteroides* group in celiac children with active disease.

The immunomodulatory properties of different *Bifidobacterium* species and strains have been reported (Young et al. 2004); furthermore, it has been shown that the administration of probiotics (e.g., bifidobacteria, Table 4) has positive effects on inflammatory and allergic bowel diseases (Dotan

and Rachmilewitz 2005). These results coupled to the microbiota analyses on children suffering from CD reported above open the possibility of using certain *Bifidobacterium* strains as adjuvant for therapeutic use for CD in children. De Angelis et al. (2006) have shown the capacity of the already mentioned probiotic preparation VLS#3 of decreasing the toxicity of gluten during food processing (De Angelis et al. 2006). Furthermore, VLS#3 has been shown to colonize the intestine and increase epithelial barrier function in the host (Kaur et al. 2002), and therefore, the mentioned probiotics mixture has the potential for being used to modify and improve gliadin degradation in the gastrointestinal tract after ingestion. This approach has been adopted by Lindfors et al. (2008) who showed, in vitro, that live *B. lactis* is able to inhibit and at least partially counter the damage induced by gliadin administration in intestinal epithelial cell lines (Caco-2 and T84). However, in vivo studies on bifidobacteria administration in celiac children are needed in order to better outline the benefits of this microbial group as adjuvant in the CD therapy in childhood.

Obesity

Obesity, a condition in which an abnormally large amount of fat is stored in the adipose tissue, resulting in an increase in body weight, is one of the major public health problems in developed countries. Although it is accepted that obesity results from disequilibrium between energy intake and expenditure, it is a complex disease and not completely understood since it involves both genetic and environmental factors. Some authors consider obesity as a transmissible disease because maternal obesity predisposes children to adulthood obesity (Lawlor et al. 2007). Nowadays, obesity prevalence is increasing especially among children and adolescents and it can be considered a worldwide epidemic. Recently, obesity has been associated with a specific profile of the bacterial gut microbiota; in particular, studies have focused not only on individual bacterial species but on the contribution of the whole microbial communities (Tennyson and Friedman 2008). However, controversial data make it clear that the connection between the microbiota composition and excess weight is very complex.

In recent experimental studies, significant associations were found between obesity and the increase of some bacterial groups such as *Lactobacillus* spp., *Staphylococcus aureus*, and *E. coli*. On the other hand, other microbial groups, such as bifidobacteria, have been associated with lean status (Million et al. 2012). Members of the genus *Bifidobacterium* were shown to be higher in number in children who remained normal weight at 7 years old than in children developing overweight (Kalliomaki et al. 2008), allowing the authors to conclude that an aberrant compositional development of the gut microbiota precedes overweight and this may offer new possibilities for preventive and therapeutic applications of bifidobacteria in weight management. Furthermore, other studies reported a decrease of total bifidobacteria in feces of obese patients (Schwiertz et al. 2010; Collado et al. 2008). A recent study analyzed the fecal concentration of principal intestinal microbial groups in obese, overweight, lean, and anorexic subjects. A positive correlation was found between certain *Lactobacillus* species, in particular *L. reuteri*, and obesity. On the contrary, *B. animalis* has been associated with a lower body mass index (Million et al. 2013). Several meta-analyses, in the past years, have shown that breastfeeding is associated with a reduced likelihood of overweight or obesity in childhood and that the duration of breastfeeding is inversely associated with the risk of overweight (Owen et al. 2005; Harder et al. 2005). Several results mainly performed on animal models suggested that some strains of bifidobacteria can have an action on lipid metabolism and may be potential therapeutic candidates for management of obesity. An early study (Xiao et al. 2003) showed that a strain of *B. longum* exhibited a significant effect in lowering serum total cholesterol (Table 4) both in rats and humans and that this effect was greater when a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was used. Another study evidenced that *Bifidobacterium* spp. are significantly and positively correlated with improved glucose tolerance, glucose-induced insulin secretion, and decreased plasma and adipose tissue proinflammatory cytokines in probiotic-treated mice (Cani et al. 2007). Finally, VLS#3, a mixture of bifidobacteria and lactobacilli already used to reduce symptoms of CD and of allergic disease, was found to improve diet-induced obesity and its related hepatic steatosis and insulin resistance by increasing hepatic natural killer T cells and reducing inflammatory signaling in mice (Ma et al. 2008). A supplement containing *B. pseudocatenulatum* SPM 1204, *B. longum* SPM 1205, and *B. longum* SPM 1207, possessing immune-modulatory properties and hypocholesteremia effects, was administered to high fat diet-induced obese rats (An et al. 2011). The administration reduced body and fat weights, fat serum levels, and harmful enzyme activities such as β-glucuronidase and tryptophanase.

The explanations for the ability of the gut microorganisms to affect obesity development include an improvement of the energy harvest from the diet, influence on lipase activity, a decrease of lipopolysaccharide (LPS) inflammation that is related to fat-induced system, and the control on endotoxemia and insulin resistance (Blaut and Bischoff 2010). Cani et al. (2009) have obtained the capability of *Bifidobacterium* spp. to improve the gut barrier and its correlation with the lower plasma LPS level and inflammatory tone. The authors have explained that the association of probiotics and prebiotic is significantly correlated with a strong decrease in markers of oxidative and inflammatory stress in liver tissue with final beneficial consequences on associated metabolic disorders.

Yin et al. (2010) have evaluated the response of glucose and lipid metabolism to the administration of several *Bifidobacterium* strains. They found that the administration of bifidobacteria were able to reduce serum and liver triglyceride levels and decrease hepatic adiposity. The response was found to be strain dependent. Therefore, the correlation between decreased proportions of bifidobacteria and an increased risk of symptoms of metabolic syndrome and overweight status seems to be an achieved result. However, oral administration of exogenous bifidobacteria against this pathologic status needs to be further investigated.

Neurological and psychiatric diseases

The brain and the gut are in constant bidirectional communication through neural pathways and immune and endocrine mechanisms. This is what has been defined as "the gut–brain axis" (Rhee et al. 2009). The GIT is a point of interaction between microorganisms, immune cells, and the neuronal network. In this respect, beneficial microbes such as lactobacilli and bifidobacteria seem to be particularly sensitive to signals from the central nervous system, taking into consideration that stressful conditions, including emotional stress, are very often accompanied by a decrease of these organisms in the GIT. These microbial alterations may be a result of changes in gut motility, increased acidity, and/or direct effects of neurochemicals. Not only microorganisms respond to the neuroendocrine environment, but also bacteria themselves can influence the endocrine system by the production of several biologically active peptide, nitric oxide, melatonin, gamma-aminobutyric acid, and serotonin (Collins and Bercik 2009). Moreover, a link between the functionality of the immune system and mood disorders has been established: properly functioning adaptive immunity is important in the maintenance of mental activity and in coping with conditions leading to cognitive deficits (Forsythe et al. 2010).

The immunomodulatory action of probiotics through the production of specific cytokines are well documented, and given the potentially important role of cytokines in mood disorders, probiotics are likely to influence brain functions by their effects on the immune system (Cryan and O'Mahony 2011). A decrease in the desirable GIT bacteria will lead to deterioration in gastrointestinal, neuroendocrine, and immune relationships and, ultimately, disease. Therefore, studies focusing on the impact of enteric microbiota on the central nervous system are essential to the understanding of the influence of this system.

One approach that is being utilized to study the microbiota–gut–brain axis is the use of germ-free mice, which offer the possibility to study the impact of the complete absence of a gastrointestinal microbiota on behavior. Researches performed with acute stressed germ-free mice, showing hyperactivity of the body major stress response system (the hypothalamic–pituitary–adrenal axis), have evidenced that the stress response was normalized by administration to mice of a *B. infantis* strain (Sudo et al. 2004). Bercik et al. (2011) have shown that infection-induced behavioral changes in mice could be reversed by *B. longum* NCC3001 strain administration. *B. infantis* 35624 strain has been shown in Sprague–Dawley rats to induce an increase of levels of plasma tryptophan, a precursor of serotonin which is a key neurotransmitter within the gut–brain axis possessing antidepressant properties (Desbonnet et al. 2008) (Table 4). Since tryptophan concentrations in the central nervous system are largely dependent on peripheral availability and the enzymatic machinery responsible for the production of serotonin is not saturated at normal tryptophan concentrations (Ruddick et al. 2006), the implication is that the microbiota might play some role in the regulation of the central and as enteric nervous system serotonin synthesis. This effect is potentially mediated by the influence of the microbiota on the expression of indoleamine-2,3-dioxygenase, a key enzyme in the physiologically dominant pathway of tryptophan degradation (Forsythe et al. 2010), but of course multiple mechanisms are possible and indeed likely, considering the strain-specific effects that have been observed in many probiotic studies to date.

The research in the neurology and psychiatry sector has scarcely reached the point of intervention studies targeted to humans and, in particular, to infants. One of the few intervention studies performed has assessed the effect of a combination of *Lactobacillus helveticus* R0052 strain and *B. longum* R0175 strain on both human subjects and rats showing that these probiotics reduced anxiety in animals and had beneficial psychological effects with a decrease in serum cortisol in patients (Messaoudi et al. 2010). The aim for future research in this field is to definitely clarify the effects of the gut microbiota on several brain-related functions in order to identify the microbial species that are critical for the development of a healthy phenotype and those that may have negative impacts on behavior, mood, and emotion in humans. This will pose the basis for targeted probiotic intervention trials.

Future perspectives

This review has outlined that the composition of the gut microbiota is strictly linked to a health status of the host. In this respect, bifidobacteria play a pivotal role considering that several pathologies, and not only enteric diseases, show a reduced level of this microbial group and, in addition, a reduced biodiversity of the species present. This is particularly true for newborn and children, who possess an unstable gut microbial composition which is more susceptible to variations caused by external factors. Due to these considerations, the use of bifidobacteria as probiotic for preventive or therapeutic agents is an established fact for some enteric diseases such as

acute diarrhea and NEC, but it can be feasible for several diseases which are not apparently linked to the GIT microbiota composition, such as obesity and neurologic diseases. Unfortunately, most of the experimental evidences for these diseases regard in vitro studies, cell line experiments and, in some cases, animal experimental model researches. When these preliminary results are consolidated, clinical intervention trials using bifidobacteria strains possessing the GRAS and the QPS status can be planned to achieve definitive results on humans.

References

Akobeng AK, Ramanan AV, Buchan I, Heller RF (2006) Effect of breast feeding on risk of coeliac disease: a systematic review and meta-analysis of observational studies. Arch Dis Child 91:39–43

Aloisio I, Santini C, Biavati B, Dinelli G, Cencič A, Chingwaru W, Mogna L, Di Gioia D (2012) Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns. Appl Microbiol Biotechnol 96:1561–1576

Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F (2011) Diarrheagenic *Escherichia coli* in acute gastroenteritis in infants in North-West Italy. New Microbiol 34:45–51

An HM, Park SY, Lee do K, Kim JR, Cha MK, Lee SW, Lim HT, Kim KJ, Ha NJ (2011) Antiobesity and lipid-lowering effects of *Bifidobacterium* spp. in high fat diet-induced obese rats. Lipids Health Dis 10:116

Arboleya S, Ruas-Madiedo P, Margolles A, Solís G, Salminen S, de Los Reyes-Gavilán CG, Gueimonde M (2011) Characterization and in vitro properties of potentially probiotic *Bifidobacterium* strains isolated from breast-milk. Int J Food Microbiol 149:28–36

Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 101:15718–15723

Barclay AR, Stenson B, Simpson JH, Weaver LT, Wilson DC (2007) Probiotics for necrotizing enterocolitis: a systematic review. J Pediatr Gastroenterol Nutr 45:569–576

Barrett E, Guinane C, Ryan A, Dempsey E, Murphy B, O'Toole P, Fizgerarld G, Cotter P, Ross P, Stanton C (2013) Microbiota diversity and stability of the preterm neonatal ileum and colon of two infants. MicrobiolOpen 2:215–225

Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, Deng Y, Blennerhassett P, Fahnestock M, Moine D, Berger B, Huizinga J, Kunze W, Mclean P, Bergonzelli G, Collins S, Verdu E (2011) The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut–brain communication. Neurogastroenterol Motil 23:1132–1139

Bezirtzoglou E, Tsiotsias A, Welling GW (2011) Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). Anaerobe 17:478–482

Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C (2010) Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev 86:13–15

Biavati B, Castagnoli P, Crociani F, Trovatelli LD (1984) Species of the *Bifidobacterium* in the feces of infants. Microbiologica 7:341–345

Biavati B, Vescovo M, Torriani S, Bottazzi V (2000) Bifidobacteria: history, ecology, physiology and applications. Ann Microbiol 50:117–131

Bingley PJ, Williams AJ, Norcross AJ, Unsworth DJ, Lock RJ, Ness AR, Jones RW (2004) Undiagnosed coeliac disease at age seven: population based prospective birth cohort study. BMJ 328:322–323

Blaut M, Bischoff SC (2010) Probiotics and obesity. Ann Nutr Metab 57:20–23

Boyle RJ, Tang ML (2006) The role of probiotics in the management of allergic diseases. Clin Exp Allergy 36:568–576

Braga TD, da Silva GA, de Lira PI, de Carvalho Lima M (2011) Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial. Am J Clin Nutr 93:81–86

Cabana MD, McKean M, Wong AR, Chao C, Caughey AB (2007) Examining the hygiene hypothesis: the trial of infant probiotic supplementation. Paediatr Perinat Epidemiol 21:23–28

Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM (2007) Selective increases of bifidobacteria in gut microflora improves high-fat diet induced diabetes in mice through a mechanism associated with endotoxemia. Diabetologia 50:2374–2383

Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM (2009) Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2 driven improvement of gut permeability. Gut 58:1091–1103

Claud EC, Walker WA (2001) Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. FASEB J 15:1398–1403

Collado MC, Calabuig M, Sanz Y (2007) Differences between the faecal microbiota of coeliac children and healthy controls. Curr Issues Intest Microbiol 8:9–14

Collado MC, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y (2008) Imbalances in faecal and duodenal *Bifidobacterium* species composition in active and non-active celiac disease. BMC Microbiol 8:232

Collins SM, Bercik P (2009) The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterology 136:2003–2014

Coppa G, Gabrielli O (2008) Human milk oligosaccharides as prebiotics. In: Versalovic J, Wilson M (eds) Therapeutic microbiology: probiotics and related strategies. American Society for Microbiology Press, Washington, pp 131–146

Corvaglia L, Legnani E, Di Gioia D, Aloisio I, Martini S, Oss M, Biavati B, Faldella G (2012) Effects of intrapartum antibiotic prophylaxis on newborn microbiota. Arch Dis Child 97:A380. doi:10.1136/archdischild-2012-302724.1334, Abstract at the 4th Congress of the European Academy of Paediatric Societies (EAPS). Istanbul, Turkey, 5–9 October 2012

Cryan JF, O'Mahony SM (2011) The microbiome–gut–brain axis: from bowel to behavior. Neurogastroenterol Motil 23:187–192

De Angelis M, Rizzello CG, Fasano A, Clemente MG, De Simone C, Silano M, De Vincenzi M, Losito I, Gobbetti M (2006) Vsl#3 probiotic preparation has the capacity to hydrolyze gliadin polypeptides responsible for Celiac Sprue. Biochim Biophys Acta 1762:80–93

de Weerth C, Fuentes S, Puylaert P, de Vos M (2013) Intestinal microbiota of infants with colic: development and specific signatures. Pediatrics 131:e550–e558

Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan T (2008) The probiotic *Bifidobacterium infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 43:164–174

Deshpande G, Rao S, Patole S (2007) Probiotics for prevention of necrotizing enterocolitis in preterm neonates with very low birthweight: a systematic review of randomized controlled trials. Lancet 369:1614–1620

Deshpande G, Rao S, Patole S, Bulsara M (2010) Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics 125:921–930

Deshpande GC, Rao SC, Keil AD, Patole SK (2011) Evidence-based guidelines for use of probiotics in preterm neonates. BMC Med 9:92

Di Felice G, Barletta B, Butteroni C, Corinti S, Tinghino R, Colombo P, Boirivant M (2008) Use of probiotic bacteria for prevention and therapy of allergic diseases: studies in mouse model of allergic sensitization. J Clin Gastroenterol 42:130–132

Dotan I, Rachmilewitz D (2005) Probiotics in inflammatory bowel disease: possible mechanisms of action. Curr Opin Gastroenterol 21: 426–430

Dubois PC, van Heel DA (2008) Translational mini-review series on the immunogenetics of gut disease: immunogenetics of coeliac disease. Clin Exp Immunol 153:162–173

EFSA (2013) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. EFSA J 11:3129, 250 pp

Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, Aguilera M, Khanna S, Gil A, Edwards CA, Doré J, INFABIO team (2010) Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr 51:77–84

Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M, Gil A, Vieites JM, Norin E, Young D, Scott JA, Doré J, Edwards CA, INFABIO team (2011) Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology 157:1385–1392

Fasano A, Catassi C (2005) Coeliac disease in children. Best Pract Res Clin Gastroenterol 19:467–478

Favier CF, Vaughan EE, De Vos WM, Akkermans AD (2002) Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68:219–226

Feleszko W, Jaworska J, Rha RD, Steinhausen S, Avagyan A, Jandzus A, Ahreus B, Gronenberg DA, Wahn V, Hamelmann E (2007) Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. Clin Exp Allergy 37:498–505

Ferrieri P, Wallen L (2012) Neonatal bacterial sepsis. In: Gleason CA, Devaskar SU (eds) Avery's disease of the newborn. Elsevier, Amsterdam, pp 538–550

Forsythe P, Sudo N, Dinan T, Taylor V, Bienenstock J (2010) Mood and gut feelings. Brain Behav Immun 24:9–16

Grandy G, Medina M, Soria R, Terán CG, Araya M (2010) Probiotics in the treatment of acute rotavirus diarrhoea. A randomized, double-blind, controlled trial using two different probiotic preparations in Bolivian children. BMC Infect Dis 10:253

Green PH, Cellier C (2007) Celiac disease. N Engl J Med 357:1731–1743

Gueimonde M, Ouwehand A, Huhtinen H, Salminen E, Salminen S (2007) Qualitative and quantitative analyses of the bifidobacterial microbiota in the colonic mucosa of patients with colorectal cancer, diverticulitis and inflammatory bowel disease. World J Gastroenterol 13:3985–3989

Hammerman C, Bin-Nun A, Kaplan M (2006) Safety of probiotics: comparison of two popular strains. BMJ 333:1006–1008

Hansen CH, Nielsen DS, Kverka M, Zakostelska Z, Klimesova K, Hudcovic T, Tlaskalova-Hogenova H, Hansen AK (2012) Patterns of early gut colonization shape future immune responses of the host. PLoS One 7:e34043

Harder T, Bergmann R, Kallischnigg G, Plagemann A (2005) Duration of breastfeeding and risk of overweight: a meta-analysis. Am J Epidemiol 162:397–403

Hunter CJ, Upperman JS, Ford HR, Camerini V (2008) Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). Pediatr Res 63:117–123

Indrio F, Neu J (2011) The intestinal microbiome of infants and the use of probiotics. Curr Opin Pediatr 23:145–150

Isolauri E (2004) Dietary modification of atopic disease: use of probiotics in the prevention ofatopic dermatitis. Curr Allergy Asthma Rep 4: 270–275

Isolauri E, Salminen S (2008) Probiotics: use in allergic disorders: a Nutrition, Allergy, Mucosal Immunology, and Intestinal Microbiota (NAMI) Research Group Report. J Clin Gastroenterol 42:S91–S96

Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S (2000) Probiotics in the management of atopic eczema. Clin Exp Allergy 30:1604–1610

Ivarsson A, Hernell O, Stenlund H, Persson LA (2002) Breast-feeding protects against celiac disease. Am J Clin Nutr 75:914–921

Kailasapathy (2008) Formulation, administration, and delivery of probiotics. In: Versalovic J, Wilson M (eds) Therapeutic microbiology: probiotics and related strategies. American Society for Microbiology, Washington, pp 97–118

Kalliomaki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E (2001) Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J Allergy Clin Immunol 107:129–134

Kalliomaki M, Collado MC, Salminen S, Isolauri E (2008) Early differences in fecal microbiota composition in children may predict overweight. Am J Clin Nutr 87:534–538

Kaur IP, Chopra K, Saini A (2002) Probiotics: potential pharmaceutical applications. Eur J Pharm Sci 15:1–9

Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, Yajima M, Dvorak B (2009) *Bifidobacterium bifidum* improves intestinal integrity in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointes Liver Physio 1297:940–949

Klaassens ES, Boesten RJ, Haarman M, Knol J, Schuren FH, Vaughan EE, de Vos WM (2009) Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants. Appl Environ Microbiol 75:2668–2676

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE (2011) Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 108:4578–4585

Kuitunen M, Kukkonen K, Juntunen-Backman K, Korpela R, Poussae T, Tuure T, Haahtela T, Savilahti E (2009) Probiotics prevent IgE-associated allergy until age 5 years in caesarean delivered children but not in the total cohort. J Allergy Clin Immunol 123: 335–341

Lawlor DA, Smith GD, O'Callaghan M, Alati R, Mamun AA, Williams GM, Najman JM (2007) Epidemiologic evidence for the fetal overnutrition hypothesis: findings from the Mater-University study of pregnancy and its outcomes. Am J Epidemiol 165:418–424

Li Y, Shimizu T, Hosaka A, Kaneko N, Ohtsuka Y, Yamashiro Y (2004) Effects of *Bifidobacterium breve* supplementation on intestinal flora of low birth weight infants. Pediatr Int 46:509–515

Lin HC, Hsu CH, Chen HL, Chung MY, Hsu JF, Lien RI, Tsao LY, Chen CH, Su BH (2008) Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. Pediatrics 122:693–700

Lindfors K, Blomqvist T, Juuti-Uusitalo K, Stenman S, Venäläinen J, Mäki M, Kaukinen K (2008) Live probiotic *Bifidobacterium lactis* bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. Clin Exp Immunol 152:552–558

Londei M, Ciacci C, Ricciardelli I, Vacca L, Quaratino S, Maiuri L (2005) Gliadin as a stimulator of innate responses in celiac disease. Mol Immunol 42:913–918

Lowenthal A, Livni G, Amir J, Samra Z, Ashkenazi S (2006) Secondary bacteremia after rotavirus gastroenteritis in infancy. Pediatrics 117: 224–226

Ma X, Hua J, Li Z (2008) Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. J Hepatol 49:821–830

Magne F, Hachelaf W, Suau A, Boudraa G, Mangin I, Touhami M, Bouziane-Nedjadi K, Pochart P (2006) A longitudinal study of infant faecal microbiota during weaning. FEMS Microbiol Ecol 58:563–571

Mai V, Young CM, Ukhanova M, Wang X, Sun Y, Casella G, Theriaque D, Li N, Sharma R, Hudak M, Neu J (2011) Fecal microbiota in premature infants prior to necrotizing enterocolitis. PLoS One 6:e20647

Meresse B, Ripoche J, Heyman M, Cerf-Bensussan N (2009) Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. Mucosal Immunol 2:8–23

Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejedi A, Bisson J, Roujeot C, PichelIn M, Cazaubiel M, Cazaubiel J (2010) Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 26:1–9

Million M, Maraninchi M, Henry F, Armougom F, Richet Carrieri H, Valero R, Raccah D, Viallettes B, Raoult D (2012) Obesity-associated gut microbiota is enriched in *Lactobacillus reuteri* and depleted in *Bifidobacterium animalis* and *Methanobrevibacter smithii*. Int J Obes 36:817–825

Million M, Angelakis E, Maraninchi M, Henry M, Giorgi R, Valero R, Vialettes B, Raoult D (2013) Correlation between body mass index and gut concentrations of *Lactobacillus reuteri*, *Bifidobacterium animalis*, *Methanobrevibacter smithii* and *Escherichia coli*. Int J Obes 37(11):1460–1467

Mohan R, Koebnick C, Schildt J, Schmidt S, Mueller M, Possner M, Radke M, Blaut M (2006) Effects of *Bifidobacterium lactis* Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study. J Clin Microbiol 44:4025–4031

Mulder CJ, Bartelsman JF (2005) Case-finding in coeliac disease should be intensified. Best Pract Res Clin Gastroenterol 19:479–486

Muñoz JA, Chenoll E, Casinos B, Bataller E, Ramón D, Genovés S, Montava R, Ribes JM, Buesa J, Fàbrega J, Rivero M (2011) Novel probiotic *Bifidobacterium longum* subsp. infantis CECT 7210 strain active against rotavirus infections. Appl Environ Microbiol 77: 8775–8783

Nadal I, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y (2007) Imbalance in the composition of the duodenal microbiota of children with coeliac disease. J Med Microbiol 56:1669–1674

Neu J, Walker WA (2011) Necrotizing enterocolitis. N Engl J Med 364: 255–264

Ouwehand AC, Isolauri E, He F, Hashimoto H, Benno Y, Salminen S (2001) Differences in *Bifidobacterium* flora composition in allergic and healthy infants. J Allergy Clin Immunol 108:144–145

Owen CG, Martin RM, Whincup PH, Smith GD, Cook DG (2005) The effect of infant feeding on the risk of obesity across the life course: a quantitative review of published evidence. Pediatrics 115:1367–1377

Özdemir Ö (2009) Gut flora development in infancy and its effect on immune system. Çocuk Enf Derg J Pediatr Inf 3:202–203

Özdemir Ö (2010) Variuos effects of different probiotic starins in allergic disorders: an update from laboratory and clinical data. Clin Exp Immunol 160:295–304

Palma GD, Capilla A, Nova E, Castillejo G, Varea V, Pozo T, Garrote JA, Polanco I, López A, Ribes-Koninckx C, Marcos A, García-Novo MD, Calvo C, Ortigosa L, Peña-Quintana L, Palau F, Sanz Y (2012) Influence of milk-feeding type and genetic risk of developing coeliac disease on intestinal microbiota of infants: the PROFICEL study. PLoS One 7:e30791

Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO (2007) Development of the human infant intestinal microbiota. PLoS Biol 5:e177

Penders J, Thijs C, van den Brandt PA, Kummeling I, Snijders B, Stelma F, Adams H, van Ree R, Stobberingh EE (2006) Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. Gut 56:661–667

Puopolo KM, Madoff LC, Eichenwald EC (2005) Early-onset group B streptococcal disease in the era of maternal screening. Pediatrics 115:1240–1246

Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain–gut–enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6:306–314

Ruddick J, Evans A, Nutt D, Lightman S, Rook G, Lowry C (2006) Tryptophan metabolism in the central nervous system: medical implications. Expert Rev Mol Med 8:1–27

Saavedra JM, Tschernia A (2002) Human studies with probiotics and prebiotics: clinical implications. Br J Nutr 87:241–246

Sanders ME, Akkermans LM, Haller D, Hammerman C, Heimbach J, Hörmannsperger G, Huys G, Levy DD, Lutgendorff F, Mack D, Phothirath P, Solano-Aguilar G, Vaughan E (2010) Safety assessment of probiotics for human use. Gut Microbes 1:164–185

Sanz Y, Sánchez E, Marzotto M, Calabuig M, Torriani S, Dellaglio F (2007) Differences in faecal bacterial communities in coeliac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. FEMS Immunol Med Microbiol 51:562–568

Sartor BR (2004) Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology 126:1620–1633

Savino F, Pelle E, Palumeri E, Oggero R, Miniero R (2007) *Lactobacillus reuteri* (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. Pediatrics 119:124–130

Savino F, Cordisco L, Tarasco V, Calabrese R, Palumeri E, Matteuzzi D (2009) Molecular identification of coliform bacteria from colicky breastfed infants. Acta Paediatr 98:1582–1588

Savino F, Cordisco L, Tarasco V, Palumeri E, Calabrese R, Oggero R, Roos S, Matteuzzi D (2010) *Lactobacillus reuteri* DSM 17939 in infantile colic: a randomized, double-blind, placebo-controlled trial. Pediatrics 126:526–533

Savino F, Cordisco L, Tarasco V, Locatelli E, Di Gioia D, Oggero R, Matteuzzi D (2011) Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC Microbial 11:157

Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, Hardt PD (2010) Microbiota and SCFA in lean and overweight healthy subjects. Obesity 18:190–195

Sisk PM, Lovelady CA, Dillard RG, Gruber KJ, O'Shea TM (2007) Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. J Perinatol 27:428–433

Soh SE, Aw M, Gerez I, Chong YS, Rauff M, Ng YPM, Wong HB, Pai N, Lee BW, Shek LPC (2009) Probiotic supplementation in the first 6 months of life in at risk Asian infants—effects on eczema and atopic sensitization at the age of 1 year. Clin Exp Allergy 39:571–578

Solís G, de Los Reyes-Gavilan CG, Fernández N, Margolles A, Gueimonde M (2010) Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe 16:307–310

Stockman JA (2009) Newborn: probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials. In: Deshpande G, Rao S, Patole S (eds) Yearbook of pediatrics. Elsevier, Philadelphia, pp 441–443

Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu X, Kubo C, Koga Y (2004) Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J Physiol 558:263–275

Sullivan S, Schanler RJ, Kim JH, Patel AL, Trawöger R, Kiechl-Kohlendorfer U, Chan GM, Blanco CL, Abrams S, Cotten CM, Laroia N, Ehrenkranz RA, Dudell G, Cristofalo EA, Meier P, Lee ML, Rechtman DJ, Lucas A (2010) An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 156:562–567

Symonds EL, O'Mahony C, Lapthorne S, O'Mahony D, Sharry JM, O'Mahony L, Shanahan F (2012) *Bifidobacterium infantis* 35624 protects against salmonella-induced reductions in digestive enzyme activity in mice by attenuation of the host inflammatory response. Clin Transl Gastroenterol 3:e15

Szajewska H, Ruszczyński M, Radzikowski A (2006) Probiotics in the prevention of antibiotic-associated diarrhea in the children: a meta-analysis of randomized controlled trials. J Pedriatr 149:367–372

Tennyson CA, Friedman G (2008) Microecology, obesity, and probiotics. Curr Opin Endocrinol Diabetes Obes 15:422–427

Tham CSC, Peh KK, Bhat R, Liong MT (2011) Probiotic properties of bifidobacteria and lactobacilli isolated from local dairy products. Ann Microbiol 62:1079–1087

Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M (2012) Diversity of bifidobacteria within the infant gut microbiota. PLoS One 7:e36957

Underwood MA, Kananurak A, Coursodon CF, Adkins-Reick CK, Chu H, Bennett SH, Wehkamp J, Castillo PA, Leonard BC, Tancredi DJ, Sherman MP, Dvorak B, Bevins CL (2012) *Bifidobacterium bifidum* in a rat model of necrotizing enterocolitis: antimicrobial peptide and protein responses. 71:546–551

van Heel DA, West J (2006) Recent advances in coeliac disease. Gut 55: 1037–1046

Vandenplas Y, De Hert SG (2011) Randomised clinical trial: the synbiotic food supplement probiotical vs. placebo for acute gastroenteritis in children. Aliment Pharmacol Ther 34:862–867

Verdu EF (2009) Probiotics effects on gastrointestinal function: beyond the gut? Neurogastroenterol Motil 21:477–480

Viljanen M, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M (2005) Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allergy 60:494–500

Wada M, Nagata S, Saito M, Shimizu T, Yamashiro Y, Matsuki T, Asahara T, Nomoto K (2010) Effects of the enteral administration of *Bifidobacterium breve* on patients undergoing chemotherapy for pediatric malignancies. Supp Care Cancer 18:751–759

Wang C, Shoji H, Sato H, Nagata S, Ohtsuka Y, Shimizu T, Yamashiro Y (2007) Effects of oral administration of *Bifidobacterium breve* on fecal lactic acid and short-chain fatty acids in low birth weight infants. J Pediatr Gastroenterol Nutr 44:252–257

Wassenaar TM, Klein G (2008) Safety aspects and implications of regulation of probiotic bacteria in food and food supplements. J Food Protect 71:1734–1741

Weizman Z, Asli G, Alsheikh A (2005) Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. Pediatrics 115:5–9

Wiegering V, Kaiser J, Tappe D, Weissbrich B, Morbach H, Girschick HJ (2011) Gastroenteritis in childhood: a retrospective study of 650 hospitalized pediatric patients. Int J Infect Dis 15:401–407

Xiao JZ, Kondo S, Takahashi N, Miyaji K, Oshida K, Hiramatsu A, Iwatsuki K, Kokubo S, Hosono A (2003) Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. J Dairy Sci 86:2452–2461

Xiao JZ, Kondo S, Yanagisawa N, Takahashi N, Odamaki T, Iwabuchi N, Miyaji K, Iwatsuki K, Togashi H, Enomoto K, Enomoto T (2006) Probiotics in the treatment of Japanese cedar pollinosis: a double-blind placebo-controlled trial. Clin Exp Allergy 36:1425–1435

Yin YN, Yu QF, Fu N, Liu XW, Lu FG (2010) Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats. World J Gastroenterol 16:3394–3401

Young SL, Simon MA, Baird MA, Tannock GW, Bibiloni R, Spencely K, Lane JM, Fitzharris P, Crane J, Town I, Addo-Yobo E, Murray CS, Woodcock A (2004) Bifidobacterial species differentially affect expression of cell surface markers and cytokines of dendritic cells harvested from cord blood. Clin Diagn Lab Immunol 11:686–690

PAPER 3

The Probiotic *Bifidobacterium breve* B632 Inhibited the Growth of *Enterobacteriaceae* within Colicky Infant Microbiota Cultures

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Research Article

The Probiotic *Bifidobacterium breve* B632 Inhibited the Growth of *Enterobacteriaceae* within Colicky Infant Microbiota Cultures

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Infant colic is a common gastrointestinal disorder of newborns, mostly related to imbalances in the composition of gut microbiota and particularly to the presence of gas-producing coliforms and to lower levels of Bifidobacteria and Lactobacilli. Probiotics could help to contain this disturbance, with formulations consisting of *Lactobacillus* strains being the most utilized. In this work, the probiotic strain *Bifidobacterium breve* B632 that was specifically selected for its ability to inhibit gas-producing coliforms, was challenged against the *Enterobacteriaceae* within continuous cultures of microbiota from a 2-month-old colicky infant. As confirmed by RAPD-PCR fingerprinting, *B. breve* B632 persisted in probiotic-supplemented microbiota cultures, accounting for the 64% of Bifidobacteria at the steady state. The probiotic succeeded in inhibiting coliforms, since FISH and qPCR revealed that the amount of *Enterobacteriaceae* after 18 h of cultivation was 0.42 and 0.44 magnitude orders lower ($P < 0.05$) in probiotic-supplemented microbiota cultures than in the control ones. These results support the possibility to move to another level of study, that is, the administration of *B. breve* B632 to a cohort of colicky newborns, in order to observe the behavior of this strain *in vivo* and to validate its effect in colic treatment.

1. Introduction

In the first hours of life, the germ-free gastrointestinal tract of newborns is colonized by microorganisms deriving from the mother and from the environment, with the establishment of a microbial community that will evolve into one of the most complex microbial ecosystems [1]. The maintenance of a correct balance of gut bacterial population is extremely important since microbiota performs a variety of activities and functions that deeply influence the health status of the host, such as the metabolism of nondigestible compounds with supply of short chain fatty acids, vitamin biosynthesis, the regulation of immune system, and the prevention of pathogen colonization [2, 3].

Despite the fact that increasing information about microbiota composition in adults is arising from metagenomics and other culture-independent approaches, the dynamics of initial colonization and evolution of the bacterial community during the first days of life are poorly understood so far [4]. In newborns, microbiota composition is variable and unstable, and the establishment of the intestinal microbiota is highly dependent on many factors, such as the mode of birth, breast or formula feeding, and antibiotic intake [5–7]. Furthermore, factors affecting the tropism and host-microbe interactions, such as intestinal pH, body temperature, bile acids, peristalsis, mucosal immune response receptors, and internal synergy, exert a pivoting role in shaping the composition of bacterial population [8, 9]. Initially, culturing studies indicated that the pioneer bacteria colonizing the digestive tract of newborns are Enterobacteriaceae and Gram-positive cocci (e.g., *Streptococcus*, *Staphylococcus*), which lower the redox potential and generate an anoxic environment, favorable for the establishment of strictly anaerobic bacteria, such as Bacteroidetes, *Bifidobacterium*, and Clostridiales [8, 10].

Bifidobacteria are generally reported to prevail in the gut microbiota of naturally delivered breast-fed infants after a few days, at the expenses of Enterobacteriaceae and facultative aerobes [11]. However, culture independent investigations have provided evidence that infant colonization may be much more complex, since it may be primed by anaerobes as well (e.g. Clostridiales) and *Bifidobacteria* may not be among the first colonizers or may remain a numerical minority [12].

Infant colic is a common functional gastrointestinal disorder of newborns, characterized by long bouts of crying and hard-to-relieve behavior [13]. Crying peaks range between 6 and 12 weeks of age and cause considerable concern and distress to parents. The pathogenesis of infant colic is not well understood, and several underlying causes have been suggested [13]. Among them, the relationship between colonic microbiota and this disorder is emerging as a major determinant. Culturing studies revealed higher counts of Gram-negative bacteria and a less numerous population of *Lactobacilli* and *Bifidobacteria* in the feces of colicky infants compared with healthy infants [14]. Molecular global investigation of the microbiota composition through phylogenetic microarray analysis demonstrated that gut microbiota differentiate much more slowly in colicky infants than in healthy ones and that colic correlated positively with the presence of specific genera of Gammaproteobacteria (such as *Escherichia*, *Klebsiella*, *Serratia*, *Vibrio*, *Yersinia*, and *Pseudomonas*) and negatively with bacteria belonging to the Bacteroidetes and Firmicutes [15, 16]. Consistently, it is known that Enterobacteriaceae, such as bacteria belonging to *Escherichia* and *Klebsiella*, produce gas from mixed acid fermentation and proinflammatory lipopolysaccharides, both these mechanisms being proposed to favor colic [17, 18].

The microbiota of colicky infants also presents lower amounts of *Bifidobacteria* and *Lactobacilli*, which are known to be anti-inflammatory and to exert various healthy properties [19–21]. The intake of probiotic *Lactobacilli* during the first months of life can contribute to containing colic [22, 23]. On the contrary, *in vivo* studies utilizing probiotic *Bifidobacteria* for the treatment of colic are lacking. The strain *Bifidobacterium breve* B632 possesses antimicrobial activity against gas-producing coliforms isolated from the stools of infants suffering from colic [24].

In order to obtain preliminary results that could support an *in vivo* trial, the present study challenged *B. breve* B632 against the Enterobacteriaceae within cultures of microbiota from a 2-month-old colicky infant. A continuous culture fermentation simulating the gut microbiota of a colicky infant was performed to examine the time-course of *E. coli* and Enterobacteriaceae populations.

2. Methods

2.1. Chemicals and Bacterial Strain. All the chemicals were supplied by Sigma (Stenheim, Germany), unless otherwise stated. *Bifidobacterium breve* B632 was obtained from BUSCoB strain collection (Scardovi Collection of *Bifidobacteria*, Dept. of Agro-Environmental Science and Technology, University of Bologna, Italy). The strain was accepted for deposit by DSMZ for patent purposes and named *B. breve* DSMZ 24706. It was cultured anaerobically at 37°C in Lactobacilli MRS broth (BD Difco, Sparks, USA) containing 0.5 g/L L-cysteine hydrochloride (hereinafter called MRS).

2.2. Cultures of Gut Microbiota. The cultures of gut microbiota were performed in a microbiota medium MM [25], where the carbon source was substituted with 6.0 g/L of a mixture of galactooligosaccharides (GOS, Domo Vivinal, Needseweg, The Netherlands) and fructooligosaccharides (FOS, Beneo-Orafti P95, Oreye, Belgium). The mixture was composed of 90% GOS and 10% FOS (w/w), in agreement with the composition of prebiotic infant formula [26]. Oligosaccharides were filter-sterilized (0.22 μm) and added to the medium after autoclaving.

Fresh feces from a breast-fed colicky infant, born by natural delivery and not treated with antibiotics or probiotics, were utilized to prepare the inoculum for single-stage continuous cultures. Inoculum preparation was performed in anaerobic cabinet under an 85% N_2, 10% CO_2, and 5% H_2 atmosphere. Feces were diluted to the ratio of 1 : 10 (w/v) in MM, supplemented with 10% glycerol (v/v), and stored at −80°C until use.

In control microbiota cultures (MC), 5 mL of fecal suspension was thawed at 37°C and utilized to inoculate benchtop bioreactors (Sixfors V3.01, Infors, Bottmingen, Swiss) containing 250 mL of MM. Fresh MM was fed at the dilution rate of 0.042 h^{-1}, corresponding to one turnover per day. The medium was flushed with CO_2 to maintain anaerobiosis. The culture was kept in anaerobiosis at 37°C, under gentle agitation. Automatic titration with 4 M NaOH maintained pH at 6.5.

In probiotic-supplemented microbiota cultures (PMC), fecal cultures were supplemented with 5.0 E + 7 cfu/mL of *B. breve* B632. Concentrated stock cultures of *B. breve* B632 were supplemented with glycerol (10%, v/v), enumerated onto MRS-agar plates, and stored at −80°C until an appropriate volume was thawed and used for bioreactor inoculation.

Samples from MC and PMC were periodically collected to analyze fermentation products, to examine the microbiota composition, and to enumerate and isolate *bifidobacteria*.

2.3. Fluorescent In Situ Hybridization (FISH). FISH enumeration of total bacteria, *bifidobacteria*, and Enterobacteriaceae was based on the procedure of Harmsen et al. [27], with slight modifications. Culture samples were diluted to the ratio of 1 : 4 with 40 g/L paraformaldehyde and incubated overnight at 4°C. Fixed cells were washed with PBS at pH 7.4 and then dehydrated with PBS-ethanol 1 : 1 solution for 1 h at 4°C. The probes Eub 338, Bif 164, and Enterobact D, were used for total bacteria, *bifidobacteria*, and Enterobacteriaceae, respectively [28]. To perform hybridization, 10 μL of cell suspension, 1 μL of the specific FITC-labeled probe, and 100 μL of hybridization buffer (20 mM TRIS-HCl, 0.9 M NaCl, and 0.1% SDS) were mixed and incubated for 16 h at the temperature specific for each probe [28].

A proper amount of the cell suspension was diluted in 4 mL of washing buffer (20 mM TRIS-HCl, 0.9 M NaCl) and

maintained at hybridization temperature for 10 min before being filtered onto 0.2 μm polycarbonate filters (Millipore, Ettenleur, The Netherlands). Filters were mounted on microscope slides with Vectashield (Vector Labs, Burlingame, California). The slides were evaluated with a fluorescence microscope (Eclipse 80i, Nikon Instruments) equipped with mercury arc lamp, FITC specific filter, and digital camera. Depending on the number of fluorescent cells, 30 to 100 microscopic fields were counted and averaged in each slide. Each sample was enumerated in triplicate.

2.4. qPCR. Biomass samples from MC and PMC cultures were collected by centrifugation, suspended in PBS (pH 7.8), and extracted with QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) to obtain bacterial gDNA. gDNA was quantified with NanoPhotometer P-Class (Implen GmbH, Munchen, Germany), diluted to 2.5 ng/μL in TE buffer pH 8, and subjected to qPCR analysis with primers targeting Enterobacteriaceae and *Escherichia coli* [29–31]. The set of primers Eco-F (GTTAATACCTTTGCTCATTGA)/Eco-R (ACCAGGGTATCAATCCTGTT) and Ent-F (ATGGCTGTCGTCAGCTCGT)/Ent-R (CCTACTTCTTTTGCAACCCACTC) were used for Enterobacteriaceae and *Escherichia coli*, respectively. The mixture contained 10 μL of SsoFast EvaGreen Supermix, 4 μL of each 2 μM primer, and 2 μL of template. qPCR reaction was carried out with the CFX96 Real-Time System (Bio-Rad Laboratories, Redmond, WA, USA), according to the following protocol: 98°C for 2 min; 45 cycles at 98°C for 0.05 min, 60°C for 0.05 min, and 95°C for 1 min; 65°C for 1 min.

2.5. RAPD-PCR Tracing of Bifidobacterium breve B632. Fresh culture samples were serially diluted in Wilkins-Chalgren anaerobe broth (Oxoid) in the anaerobic cabinet and plated on RB selective medium, in order to count and isolate *Bifidobacteria* [32]. Genomic DNA was extracted from 200 colonies isolated from the PMC processes, using Instagene matrix (Bio-Rad). RAPD-PCR was carried out in a 15 μL reaction mixture: 10X Dream Taq Buffer (including $MgCl_2$ 2 mM), 1.5 μL; dNTPs mixture 0.10 mM, 0,15 μL; 2 μM M13 primer (GAGGGTGGCGGTTCT), 3.75 μL; genomic DNA, 3 μL; and PCR water 5.25 μL. DNA amplification was performed with the following protocol: 94°C for 4 min (1 cycle), 94°C for 1 min, 34°C for 1 min, 72°C for 2 min (45 cycles); 72°C for 7 min (1 cycle). The PCR products were electrophoresed in a 2% agarose gel (25 × 25 cm) for 4 h at a constant voltage (160 V) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, and pH 8.0). RAPD-PCR profiles were visualized under ultraviolet light after staining with ethidium bromide, followed by digital image capturing. The resulting fingerprints were analyzed by the Gene Directory 2.0 (Syngene, UK) software package. The similarity among digitalized profiles was calculated and a dendrogram was derived with an unweighted pair-group method using arithmetic means (UPGMA).

2.6. Analysis of Fermentation Products. The samples were clarified through centrifugation (13,000 ×g, 5 min, 4°C) and filtration (0.22 μm cellulose acetate filter) and stored at −20°C until analyzed. Fermentation products (formic, acetic, lactic, propionic, butyric, and succinic acids and ethanol) were analyzed using a HPLC device (Agilent technologies, Waldbronn, Germany) equipped with refractive index detector and Aminex HPX-87 H ion exclusion column. Isocratic elution was carried out with 0.005 M H_2SO_4 at 0.6 mL/min [33].

2.7. Statistical Analysis. All values are means of four separate experiments. Comparisons were carried out according to Student's t-test. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. Evolution of Fecal Microbial Groups and Fermentation Products. Single-stage continuous fermentation of the colonic microbiota from a colicky newborn was carried out for 24 h to study whether the addition of *B. breve* B632 could affect the growth of Enterobacteriaceae. *Bifidobacteria*, Enterobacteriaceae, and total bacteria were enumerated in MC and in PMC, the latter supplemented with 5.0 E + 07 cfu/mL of *B. breve* B632 (Figures 1(a) and 1(b)). After 18 h of cultivation, FISH bacterial counts became steady in both MC and PMC cultures. Eubacteria increased up to 9.0–9.4 E+ 09 cfu/mL, without statistically significant difference between PMC and MC ($P > 0.05$). At all the time points, *bifidobacteria* were more abundant in PMC than in MC ($P < 0.05$). Enterobacteriaceae were negatively affected by the presence of *B. breve* B632 and were always less numerous in PMC than in MC ($P < 0.05$).

The evolution of Enterobacteriaceae and *E. coli* was determined also with q-PCR during the whole process. Enterobacteriaceae were significantly lower in PMC than in MC ($P < 0.05$), consistently with FISH results. On the other hand, statistically significant difference was not observed in the levels of *E. coli* ($P > 0.05$), with the exception of 18 h, when *E. coli* was less numerous in MC than in PMC (Figure 2).

The presence of *B. breve* B632 in PMC cultures was traced using RAPD-PCR fingerprinting at all the time points. Colonies were isolated using the *Bifidobacterium* selective medium RB and those positive to *Bifidobacterium*-specific PCR were subjected to RAPD-PCR analysis. At the beginning of the fermentation, *B. breve* B632 represented the 85% of bifidobacterial isolates in PMC, then decreased to 73% after 6 h, and stabilized at 64% at the steady state ($n = 4$, SD < 34%). The relative amount of *B. breve* B632 tended to decrease, albeit differences at the diverse time points were not statistically significant. Considering that at the steady state Bifidobacteria accounted for approximately 38% of total eubacteria according to FISH enumeration, *B. breve* B632 can be estimated as approximately the 24% of total bacterial population in PMC. In these samples, 2 biotypes of *Bifidobacteria* represented the autochthonous component. The same two biotypes were identified also at the inoculum in MC cultures, together with two other minor ones, none of them exhibiting a RAPD-PCR profile similar to that of *B. breve* B632.

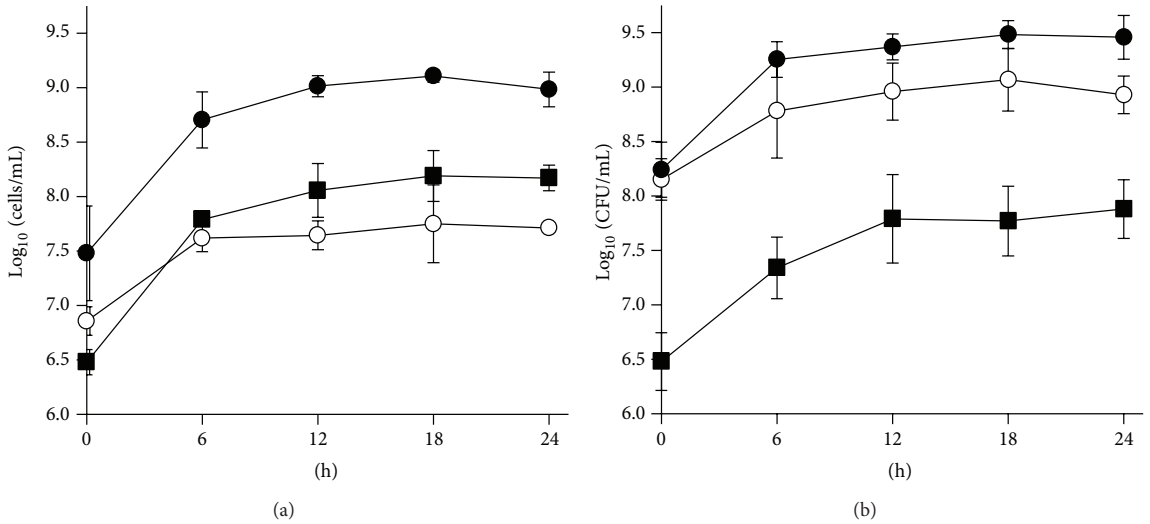

Figure 1: Time-course of total bacteria, *bifidobacteria*, and Enterobacteriaceae in cultures of infant gut microbiota. Eubacteria (●), *Bifidobacterium* (○), and Enterobacteriaceae (■) were quantified by FISH in control cultures (MC, (a)) and in cultures supplemented with *B. breve* B632 (PMC, (b)). Data are means ± SD, $n = 4$.

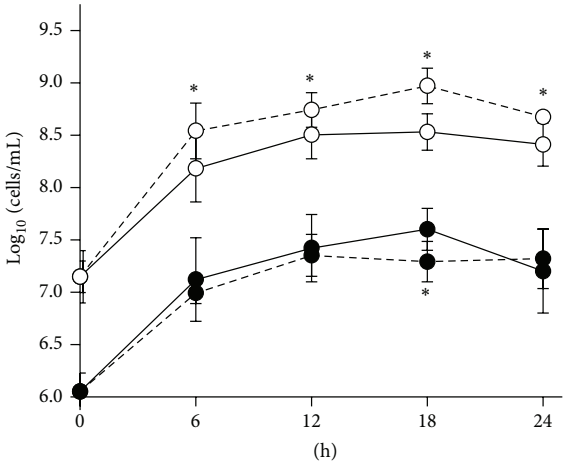

Figure 2: Time-course of *E. coli* and Enterobacteriaceae in cultures of infant gut microbiota. *E. coli* (●) and Enterobacteriaceae (○) were quantified by qPCR in control cultures (MC, dashed line) and in cultures supplemented with *B. breve* B632 (PMC, solid line). Data are means ± SD, $n = 4$. Stars indicate statistically significant difference between MC and PMC cultures ($P < 0.05$).

Formate, acetate, lactate, propionate, butyrate, and ethanol originated by microbiota metabolism during the processes (Figures 3(a) and 3(b)). Like the bacterial counts, the concentrations of microbial products became stationary after approximately 18 h. Ethanol, formate, lactate, and acetate were the first to increase at the beginning of the fermentation. Propionate, 2,3-butanediol, and butyrate accumulated later, while lactate decreased as the steady state was approached.

During the growth phase, the major differences between MC and PMC processes were acetate and ethanol, accumulating at different levels during the first hours of the process: after 12 h, in MC and PMC, ethanol was 1.6 and 0.8 g/L, while acetate 0.8 and 2.4 g/L, respectively. At the steady state (18 h), MC had higher levels of butyrate and ethanol than PMC, while acetate and lactate were higher in PMC ($P < 0.05$). The other metabolites exhibited similar steady-state concentrations in PMC and MC processes ($P > 0.05$).

4. Discussion

Literature reports the use of *Lactobacillus* spp. strains to alleviate the symptoms of infant colic [22, 23]. On the other hand, no information is available on this specific use of *bifidobacteria*, although *in vitro* results showed that strains of *Bifidobacterium* can exert antimicrobial activity against gas forming coliforms [24]. Among a panel of *Bifidobacterium* strains that were selected as potential candidates for probiotic use against colic in infants, *B. breve* B632 appeared particularly promising because of its strong antimicrobial activity against coliforms, coupled to the lack of transmissible antibiotic resistance traits and cytotoxicity for the gut epithelium. Moreover, the strain is capable of adhering to gut epithelium cell lines and could stimulate gut health by increasing metabolic activity and immune response of epithelial cells [24].

In the present work, the antagonistic effect of *B. breve* B632 against coliforms was challenged within gut microbiota cultures of a colicky newborn, simulating *in vivo* conditions, in order to propose its use as anticolic probiotic. *B. breve* B632 survived well within the fecal culture, exhibiting a high viability during the process. At all the time points, Enterobacteriaceae were significantly less numerous in presence of the probiotic. These results indicate that *B. breve* B632 exerted

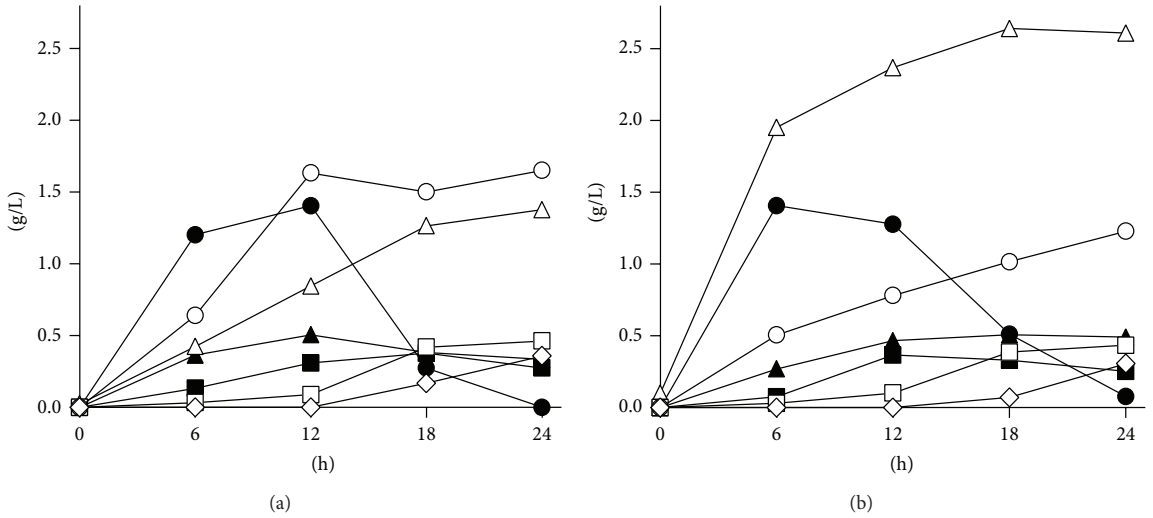

Figure 3: Time-course of fermentation products in cultures of infant gut microbiota. Ethanol (○), lactate (●), acetate (△), formate (▲), propionate (□), 2,3-butanediol (■), and butyrate (22C4) were determined in control cultures (MC, (a)) and in cultures supplemented with *B. breve* B632 (PMC, (b)). Data are means, $n = 4$, and SD always < 0.25 g/L.

antimicrobial activity against coliforms in fecal cultures as well, consistently with previous observation with spot agar tests and cocultures [24].

Unlike Enterobacteriaceae, *E. coli* counts were not affected by the presence of the probiotic. This observation can be ascribed to the different specificity of the primer sets utilized in qPCR quantification, since the primers for Enterobacteriaceae recognize a broader spectrum of species than the ones for *E. coli* (Table 1).

Based on the list of species that align with qPCR primers and FISH probes, it is likely that Gammaproteobacteria other than *E. coli* are involved in infant colic. For example, the qPCR primers for Enterobacteriaceae should recognize *Yersinia*, whereas the FISH probe for Enterobacteriaceae is expected to miss it.

Fecal samples have a microbial composition that does not exactly correspond to that of the colonic content, where major microbial-host interactions occur, and richness and diversity seem underrepresented [34]. However, systems as the one herein described are currently the best tools to investigate the external factors that could influence the intestinal microbial composition such as antibiotics or to test novel potential probiotics, before carrying out expensive *in vivo* trials. The data herein presented indicate that the potential probiotic strain *B. breve* B632 was able to survive in a complex microbial environment and restrained Enterobacteriaceae population.

Table 1: Genera of human intestinal bacteria potentially recognized by FISH probes and qPCR primers, according to SILVA.

Probe or primer set	Genus
Enterobact D	*Citrobacter*
	Cronobacter
	Edwardsiella
	Enterobacter
	Escherichia
	Klebsiella
	Kluyvera
	Pantoea
	Raoultella
	Serratia
	Shigella
Ent-F/Ent-R	*Edwardsiella*
	Escherichia
	Klebsiella
	Pantoea
	Proteus
	Providencia
	Pseudomonas
	Shigella
	Yersinia
Eco-F/Eco-R	*Cronobacter*
	Escherichia
	Shigella

5. Conclusions

The present study demonstrated the ability of a properly selected probiotic *Bifidobacterium* strain *B. breve* B632 to inhibit the growth of Enterobacteriaceae in an *in vitro* model system simulating the intestinal microbiota of a 2-month-old colicky infant. These results support the possibility to move to another level of study, that is, the administration of *B. breve* B632 to a cohort of colicky newborns, in order to observe the behavior of this strain *in vivo* and to validate its effect in colic treatment.

Conflict of Interests

The authors certify that there is no actual or potential conflict of interests in relation to this paper.

References

[1] L. Morelli, "Postnatal development of intestinal microflora as influenced by infant nutrition," *Journal of Nutrition*, vol. 138, no. 9, pp. 1791S–1795S, 2008.

[2] F. Guarner and J. R. Malagelada, "Gut flora in health and disease," *The Lancet*, vol. 361, no. 9356, pp. 512–519, 2003.

[3] I. Sekirov, S. L. Russell, L. Caetano M Antunes, and B. B. Finlay, "Gut microbiota in health and disease," *Physiological Reviews*, vol. 90, no. 3, pp. 859–904, 2010.

[4] D. di Gioia, I. Aloisio, G. Mazzola, and B. Biavati, "Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants," *Applied Microbiology and Biotechnology*, vol. 98, no. 2, pp. 563–577, 2014.

[5] M. G. Dominguez-Bello, E. K. Costello, M. Contreras et al., "Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 26, pp. 11971–11975, 2010.

[6] E. Bezirtzoglou, A. Tsiotsias, and G. W. Welling, "Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH)," *Anaerobe*, vol. 17, no. 6, pp. 478–482, 2011.

[7] F. Fouhy, R. P. Ross, G. F. Fitzgerald, C. Stanton, and P. D. Cotter, "Composition of the early intestinal microbiota: knowledge, knowledge gaps and the use of high-throughput sequencing to address these gaps," *Gut Microbes*, vol. 3, no. 3, pp. 203–220, 2012.

[8] R. I. Mackie, A. Sghir, and H. R. Gaskins, "Developmental microbial ecology of the neonatal gastrointestinal tract," *The American Journal of Clinical Nutrition*, vol. 69, no. 5, pp. 1035S–1045S, 1999.

[9] L. V. Hooper, D. R. Littman, and A. J. Macpherson, "Interactions between the microbiota and the immune system," *Science*, vol. 336, no. 6086, pp. 1268–1273, 2012.

[10] C. F. Favier, E. E. Vaughan, W. M. de Vos, and A. D. L. Akkermans, "Molecular monitoring of succession of bacterial communities in human neonates," *Applied and Environmental Microbiology*, vol. 68, no. 1, pp. 219–226, 2002.

[11] K. Orrhage and C. E. Nord, "Factors controlling the bacterial colonization of the intestine in breastfed infants," *Acta Paediatrica, Supplement*, vol. 88, no. 430, pp. 47–57, 1999.

[12] Y. Vallès, M. J. Gosalbes, L. E. de Vries, J. J. Abellán, and M. P. Francino, "Metagenomics and development of the gut microbiota in infants," *Clinical Microbiology and Infection*, vol. 18, no. 4, pp. 21–26, 2012.

[13] M. L. Cirgin Ellett, "What is known about infant colic?" *Gastroenterology Nursing*, vol. 26, no. 2, pp. 60–65, 2003.

[14] F. Savino, F. Cresi, S. Pautasso et al., "Intestinal microflora in breastfed colicky and non-colicky infants," *Acta Paediatrica*, vol. 93, no. 6, pp. 825–829, 2004.

[15] C. de Weerth, S. Fuentes, and W. M. de Vos, "Crying in infants: on the possible role of intestinal microbiota in the development of colic," *Gut Microbes*, vol. 4, no. 5, pp. 416–421, 2013.

[16] C. de Weerth, S. Fuentes, P. Puylaert, and W. M. de Vos, "Intestinal microbiota of infants with colic: development and specific signatures," *Pediatrics*, vol. 131, no. 2, pp. e550–e558, 2012.

[17] F. Savino, L. Cordisco, V. Tarasco, R. Calabrese, E. Palumeri, and D. Matteuzzi, "Molecular identification of coliform bacteria from colicky breastfed infants," *Acta Paediatrica*, vol. 98, no. 10, pp. 1582–1588, 2009.

[18] F. Savino, L. Cordisco, V. Tarasco et al., "Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants," *BMC Microbiology*, vol. 11, article 157, 2011.

[19] T. A. Oelschlaeger, "Mechanisms of probiotic actions—a review," *International Journal of Medical Microbiology*, vol. 300, no. 1, pp. 57–62, 2010.

[20] M. Rossi, A. Amaretti, A. Leonardi, S. Raimondi, M. Simone, and A. Quartieri, "Potential impact of probiotic consumption on the bioactivity of dietary phytochemicals," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 40, pp. 9551–9558, 2013.

[21] M. Rossi, A. Amaretti, and S. Raimondi, "Folate production by probiotic bacteria," *Nutrients*, vol. 3, no. 1, pp. 118–134, 2011.

[22] F. Savino, L. Cordisco, V. Tarasco et al., "*Lactobacillus reuteri* DSM 17938 in infantile colic: a randomized, double-blind, placebo-controlled trial," *Pediatrics*, vol. 126, no. 3, pp. e526–e533, 2010.

[23] J. Anabrees, F. Indrio, B. Paes, and K. Alfaleh, "Probiotics for infantile colic: a systematic review," *BMC Pediatrics*, vol. 13, p. 186, 2013.

[24] I. Aloisio, C. Santini, B. Biavati et al., "Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns," *Applied Microbiology and Biotechnology*, vol. 96, no. 6, pp. 1561–1576, 2012.

[25] F. Tomas-Barberan, R. García-Villalba, A. Quartieri et al., "In vitro transformation of chlorogenic acid by human gut microbiota," *Molecular Nutrition and Food Research*, vol. 58, no. 5, pp. 1122–1131, 2014.

[26] E. Bruzzese, M. Volpicelli, V. Squeglia et al., "A formula containing galacto- and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: an observational study," *Clinical Nutrition*, vol. 28, no. 2, pp. 156–161, 2009.

[27] H. J. M. Harmsen, P. Elfferich, F. Schut, and G. W. Welling, "A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization," *Microbial Ecology in Health and Disease*, vol. 11, no. 1, pp. 3–12, 1999.

[28] A. W. Walker, S. H. Duncan, E. C. M. Leitch, M. W. Child, and H. J. Flint, "pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon," *Applied and Environmental Microbiology*, vol. 71, no. 7, pp. 3692–3700, 2005.

[29] T. Rinttilä, A. Kassinen, E. Malinen, L. Krogius, and A. Palva, "Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR," *Journal of Applied Microbiology*, vol. 97, no. 6, pp. 1166–1177, 2004.

[30] E. Malinen, A. Kassinen, T. Rinttilä, and A. Palva, "Comparison of real-time PCR with SYBR green I or 5′-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria," *Microbiology*, vol. 149, no. 1, pp. 269–277, 2003.

[31] M. Castillo, S. M. Martín-Orúe, E. G. Manzanilla, I. Badiola, M. Martín, and J. Gasa, "Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR," *Veterinary Microbiology*, vol. 114, no. 1-2, pp. 165–170, 2006.

[32] R. Hartemink, B. J. Kok, G. H. Weenk, and F. M. Rombouts, "Raffinose-Bifidobacterium (RB) agar, a new selective medium

for bifidobacteria," *Journal of Microbiological Methods*, vol. 27, no. 1, pp. 33–43, 1996.

[33] A. Amaretti, T. Bernardi, A. Leonardi, S. Raimondi, S. Zanoni, and M. Rossi, "Fermentation of xylo-oligosaccharides by *Bifidobacterium adolescentis* DSMZ 18350: kinetics, metabolism, and β-xylosidase activities," *Applied Microbiology and Biotechnology*, vol. 97, no. 7, pp. 3109–3117, 2013.

[34] A. Durbán, J. J. Abellán, N. Jiménez-Hernández et al., "Assessing gut microbial diversity from feces and rectal mucosa," *Microbial Ecology*, vol. 61, no. 1, pp. 123–133, 2011.

PAPER 4

Development of synbiotic products for newborns and infants

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Abstract

The capability of ten commercial fibers of selectively stimulating the growth of four *Bifidobacterium* strains were studied with the purpose of developing a synbiotic product for infants. Two galactooligosaccharides (GOS), one fructooligosaccharide (sc-FOS), four inulins with different polymerization degree (DP), a glucooligosaccharide, an arabinogalactan and a hydrolysed guar gum were used (10 g l^{-1}). The prebiotic score was calculated comparing the capability of the fibers of stimulating the growth of bifidobacteria compared to potential infant pathogens. GOS, sc-FOS, low DP inulin (oligofructose) and the glucooligosaccharide could stimulate growth. However, the fibers showing the highest prebiotic score were oligofructose (Orafti®HIS), sc-FOS (Actilight®950P) and the GOS Vivinal®. Lyophilized bifidobacteria strain survival in simulated gastro-intestinal conditions was also assayed to define suitable ways of administration. Survival in gastric juice at pH 2.5 was poor, whereas it was higher at pH 4, a value closer to newborn pH. Microencapsulation in a lipid matrix ensured strain survival also at pH 2.5. Survival to 1 g l^{-1} bile salts was acceptable. The results allowed to conclude that *Bifidobacterium breve* B632 strain, in a lyophilized or microencapsulated form, has the requisites for use in synbiotic products targeted to infants coupled to a mixture of GOS and FOS or oligofructose.

KEY WORDS: prebiotics; probiotics; synbiotics; gastric resistance; microencapsulation

1. Introduction

Probiotics and prebiotics constitute a central growing market for the food/pharmaceutical industry and this has driven a lot of research aimed at understanding their activity (Kumar et al., 2014). The children associated market is of great relevance, because infants are very susceptible to diseases and non-chemotherapeutic treatments are particularly looked forward for this target group (Mugambi, Young, & Blaauw, 2014). Moreover, the baby food industry is particularly interested in probiotics and prebiotics with the aim of improving the quality of formula milks and post-weaning milks. The health status of the gut in infants is extremely important for the well-being of the whole organism in the successive stages of life (Bischoff, 2011). The stimulation of beneficial bacteria by prebiotic fibers in the infant gut is essential because these microbes, mainly belonging to the *Bifidobacterium* and *Lactobacillus* genera, play useful roles in prevention of disease (Turroni et al., 2012; Di Gioia, Aloisio, Mazzola, & Biavati, 2014). In this context, the development of synbiotic formulas, i.e. mixtures of prebiotics and probiotics, in which the prebiotic compound sustain the growth of the probiotic microorganism(s) supplied or of other beneficial bacteria in the host (Slavin, 2013), has a central role in infant nutrition. On the contrary, the growth of potential pathogenic or harmful microbes should not be enhanced by the metabolization of the fiber (Huebner, Wehling, & Hutkins, 2007). A number of works have developed and used a quantitative measure of the prebiotic efficacy of selected fibers with the use of a score, referred to as prebiotic score (Huebner et al., 2007; Marotti et al., 2012), which compares the extent to which a fiber supports selective growth of beneficial bacteria with respect to growth on glucose (i.e. an easily metabolized substrate) and to the growth of potential pathogenic bacteria. Potential pathogens to be used in this evaluation have to be selected considering the target for the prebiotic fiber under study.

The ability of bifidobacteria to utilize a large variety of oligosaccharides make them able to adapt and compete in an environment with changing nutritional conditions such as the infant gut. A recent work has allowed the identification of three *Bifidobacterium breve* and one *Bifidobacterium longum* subsp. *longum* strains as potential probiotic strains for the treatment of enteric disorders in newborns such as infantile colics or as preventive agents for infantile diarrhea of bacterial origin (Aloisio et al., 2012). These strains possess strong antimicrobial activity against coliforms and other pathogenic bacteria, do not possess transmissible antibiotic resistance traits and are not cytotoxic for the gut epithelium. In addition, the capability of one of these strains, namely *B. breve* B632, of reducing the amount of gas forming coliforms has also

been shown in an *in vitro* slurry model system simulating the intestinal microbiota of a 2-month-old colicky infant (Simone, Gozzoli, & Quartieri, 2014).

In this work, several commercial fibers, including fibers usually employed in the human diet but also less commonly used plant derived oligosaccharides, have been assayed for their capability of selectively stimulating the growth of the previously selected *Bifidobacterium* strains with the aim of developing a synbiotic product for infants. Moreover, the survival of the same strains in simulated gastro-intestinal conditions has been checked in order to define suitable ways of administering them to newborns and infants with the aim of planning a validation clinical trial.

2. Materials and Methods

2.1. Strains and culture conditions

Four *Bifidobacterium* strains (*B. breve* B632, B2274, B7840 and *B. longum* B1975), selected in a previous work (Aloisio et al., 2012) as potential probiotics for the treatments of enteric disorders in newborns, were used. *Bifidobacterium* strains were grown on Tryptone, Phytone, Yeast extract (TPY) broth (tryptone, 10 g l^{-1}, soy peptone, 5 g l^{-1}, glucose, 10 g l^{-1}, yeast extract, 2.5 g l^{-1}, K_2HPO_4, 1.5 g l^{-1}, $MgCl_2.6H_2O$, 0.5 g l^{-1}, Cystein-HCl, 0.5 g l^{-1}, Tween 80, 0.5 g l^{-1}, pH 6.5). The medium was modified to perform the growth experiment with potential prebiotic fibers. The modified medium (m-TPY) did not contain the carbon source (glucose), which was provided by the selected fiber, and had a halved amount of potential growth substrate, such as tryptone, peptone and yeast extract.

Escherichia coli ATCC 25645, two gas-forming coliforms isolated from colicky infants feces, i.e. *Klebsiella pneumoniae* GC23a and *Enterobacter cloacae* GC6a (Savino et al., 2011), and *Clostridium difficile* M216, isolated from hospitalized patients (unpublished results), were used as potential pathogen strains. *E. coli*, *K. pneumoniae* and *E. cloacae* strains were grown on M9 medium (Howard-Flanders & Theriot, 1966) with 10 g l^{-1} glucose, whereas Reinforced Clostridium Medium (RCM, Merck, Darmstadt, Germany) was used for *C. difficile.* Modified M9 medium (m-M9) for prebiotic activity tests did not contain glucose. The modified RCM broth (m-RCM) was prepared with half of the original concentration of peptone and yeast extract and no glucose. Inoculated cultures (20 ml l^{-1} inoculum) were incubated at 37° C for 24-48 h under anaerobic conditions, obtained using an anaerobic atmosphere generation system (Anaerocult® A, Merck).

2.2. Evaluation of the prebiotic activity of the fibers

2.2.1 Commercial fibers used in the study

Two galactooligosaccharides (GOS), one fructooligosaccharide (FOS), four inulins having a different degree of polymerization (DP), a glucooligosaccharide, an arabinogalactan and a partially hydrolysed guar gum were used. Available information on the fibers used, including composition and DP, the origin, the commercial name of fibers as well as the provider, are listed in Table 1.

Table 1 Commercial fibers used to evaluate the prebiotic activity versus bifidobacteria

Carbohydrate type	**Composition and degree of polymerization (DP)** (where available)	**Origin/method of manufacture**	**Commercial name of the fiber***	**Provider**
Galactooligosaccharide	GOS 59% Lactose 21% Glucose 19% Galactose 1% DP n.a.	Synthesized from lactose	Vivinal ®[1]	Domo, Netherlands
Galactooligosaccharide	Composition: n.a. DP 3 to 6	Synthesized from lactose	CUP-Oligo®[2]	Azelis SpA, Milano, Italy
Short chain-Fructooligosaccharide	Fructosyl nistose 11.3%, Nistose 42.5%, 1-Kestose 43.1%, Sucrose 2.4 % DP 2 to 5	Biosynthesis from beet sugars	Actilight®950P[3]	Beghin-Meiji, Francia
High soluble inulin (oligofructose)	Inulin 86% other sugars 14 % DP < 10	Chicory	Orafti®HSI[4]	Beneo-Orafti, Belgium
1:1 blend of oligofructose and inulin	oligofructose 92% other sugars 8 % DP N/A	Chicory	Orafti® Synergy1[4]	Beneo-Orafti, Belgium
Inulin	inulin 100% DP > 23	Chicory roots	Orafti® RaftilineHP[4]	Beneo-Orafti, Belgium
Inulin	DP 9 to 12	Chicory	Frutafit®[5]	Sensus, Netherlands
α-glucooligosaccharide	DP>3 (About 60% of the product has a DP≥ 5)	Enzymatic synthesis from maltose using a glucosyl transferase from a of *Leuconostoc mesenteroides* strain[6]	BioEcolians®[6]	Solabia group, Pantin Cedex, France
Arabinogalactan	Mixture of arabinogalactans of molecular weights (MW) between 10000 and 100000; the 100000 MW fraction constitutes about 2/3 of total weight	*Larix occidentalis*	Arabinex®[7]	Thorne research, Dover, USA
Partially hydrolysed guar gum (PHGG)	n.a.	Enzymatic hydrolysis of guar gum	Benefibra®[8]	Novartis Pharma Spa, Origgio (Va), Italy

*More information about the products are available online at the following websites:

[1] www.vivinalgos.com

[2] www.kowa-europe.com/food/

[3] www.beghin-meiji.com/actilight

[4] www.orafti.com

[5] www.sensus.us

[6] www.solabia.fr/Solabia/SolabiaNutrition.nsf/
[7] thorne.com/Products
[8] www.benefibra.it

2.2.2 Prebiotic activity assays and prebiotic score

Prebiotic activity of the assayed fibers was evaluated according to a modification of Marotti et al., (2012) by evaluating the capability of the fiber of stimulating bifidobacteria with respect to potentially pathogenic strains. Growth of bifidobacteria on the fibers was determined in m-TPY with 10 g l^{-1} of each fiber. As potential pathogenic strains, a 1:1:1 mixture of *E. coli* ATCC 25645, *K. pneumoniae* GC23a and *E. cloacae* GC6a (referred to as enteric mixture) and a culture of *C. difficile* M216 were used. The enteric mixture was prepared by growing each strain separately on m-M9 with 10 g l^{-1} glucose and then mixing the cultures in a 1:1:1 ratio. The mixture was inoculated (20 ml l^{-1}) in m-M9 with 10 g l^{-1} prebiotic fiber as the carbon source. A culture of *C. difficile* (A_{620} 0.6) was prepared in m-RCM broth with 10 g l^{-1} glucose and inoculated (20 ml l^{-1}) in m-RCM broth supplemented with 10 g l^{-1} prebiotic fiber. To quantify growth occurring from indigenous carbon sources present in the modified medium, strains were also grown on the modified media with no added carbon source. Positive growth control was prepared in the modified media with 10 g l^{-1} glucose. The prebiotic activity assay was performed in 96 well plates, which, once inoculated, were incubated at 37 °C under anaerobic conditions. The bacterial growth was determined by measuring A_{620} after 0, 6, 24, 30 and 48 hrs of incubation in a microwell plate reader (Multiskan, Thermo Electron, Oy, Vantaa, Finland). Each assay was replicated three times. The growth curves for *Bifidobacterium* strains, for the enteric mixture and for *C. difficile* grown in the presence of tested prebiotic fibers were generated by plotting the average A_{620} versus incubation time. The prebiotic score (PS) was calculated as follows (Marotti et al., 2012):

PS = {(A_{620} of *Bifidobacterium* strain on the fiber at 24 h – A_{620} nm of *Bifidobacterium* strain on the fiber at 0 h) / A_{620} of *Bifidobacterium* strain on glucose at 24 h – A_{620} of *Bifidobacterium* strain on glucose at 0 h)} – {(A_{620} nm of enteric mixture or *C. difficile* strain on the fiber at 24 h – A_{620} of enteric mixture on the fiber at 0 h)/ (A_{620} of enteric mixture on glucose at 24 h – A_{620} of enteric mixture on glucose at 0 h)}.

Factorial ANOVA was applied to analyze prebiotic scores using the Statistica Software (ver. 8.0, StatSoft, Tulsa, OK, USA). Values of $P < 0.05$ were considered to be significant.

2.3. Evaluation of strain survival under simulated intestinal conditions:

Survival of *Bifidobacterium* strains was checked in human gastric juice, kindly provided in a lyophilized form by Probiotical SpA, Novara, Italy, and suspended in sterile water to obtain its original volume. Gastric juice was used at its pH (about 2.5) and at pH increased to 4 with NaOH 0.1 mol l^{-1} to simulate the newborn gastric pH (Kageyama, 2002). Gastric juice was filter sterilized through a 0.2 μm cellulose acetate membrane (Millipore, Carrigtwohill, Ireland). In order to simulate duodenal conditions, 1 g l^{-1} porcine bile salts (Oxgal, Sigma-Aldrich) at pH 7 were used. This solution was also filter-sterilized as already described. To perform the survival assays, 0.1 g of lyophilized *Bifidobacterium* strains (nominal concentration 10^9 CFU g^{-1}) were suspended in 0.9 ml of gastric or bile juice solutions and vortexed for 20 s. All tests were conducted in sterile glass tubes. The cell suspension was incubated anaerobically at 37 °C. Cells suspended in PBS at pH 7.0 served as controls. Experiments were done in triplicate. Furthermore the survival to gastric juice at pH 2.5 of *B. breve* B632 strain in a microencapsulated form (provided by Probiotical S.p.A.) was assayed. Microencapsulation was performed in a vertical fluid bed drier Glatt GPCG2 LabSystem. A lyophilized culture of the strain was prepared with a concentration of 3.2×10^9 CFU g^{-1}. 60 g of lyophilized culture were mixed with 90 g of polyglycerol esters of saturated fatty acids. The final concentration of the microencapsulated cells was 10^9 CFU g^{-1}.

Enumeration was performed after sampling 100 μl immediately after mixing the free cells with gastric juice or bile solution (time 0) and at predetermined time intervals (30 and 60 min for simulated gastric conditions and 60 and 120 min for bile salts). The sampled amount was mixed with 900 μl of PBS, serially diluted in PBS and plated on TPY agar. In the case of microencapsulated cells, an alkaline borate buffer (pH 8.4) was used to perform the initial dilution of the cells (1:10) in order to promote the dissolution of the coating material (Lian, Hsiao, & Chou, 2003). After 10 min incubation in the alkaline buffer, further serial dilutions were performed and plated.

Mean values and the standard deviation were calculated from the data obtained from triplicate trials. Statistica Software was used for analysis of variance. A 95% confidence level was used for Duncan's, one-way significance test.

3. Results

3.1. Evaluation of the prebiotic activity of the fibers

Growth tests were initially performed on two GOS (Vivinal® and CUP-Oligo®) and 1 sc-FOS (Actilight®950P), which are fibers commonly used in infant formulas (Fig. 1). Then, 4 fibers commercialized as inulins were assayed (Fig. 1): Orafti®HSI, which has a DP < 10 and can therefore be classified as an oligofructose, Orafti®Synergy1, i.e. 1:1 blend of oligofructose and inulin whose DP is not available, Frutafit®, i.e. a low molecular weight (MW) inulin, and Orafti®RaftilineHP, a high MW inulin (DP > 23).

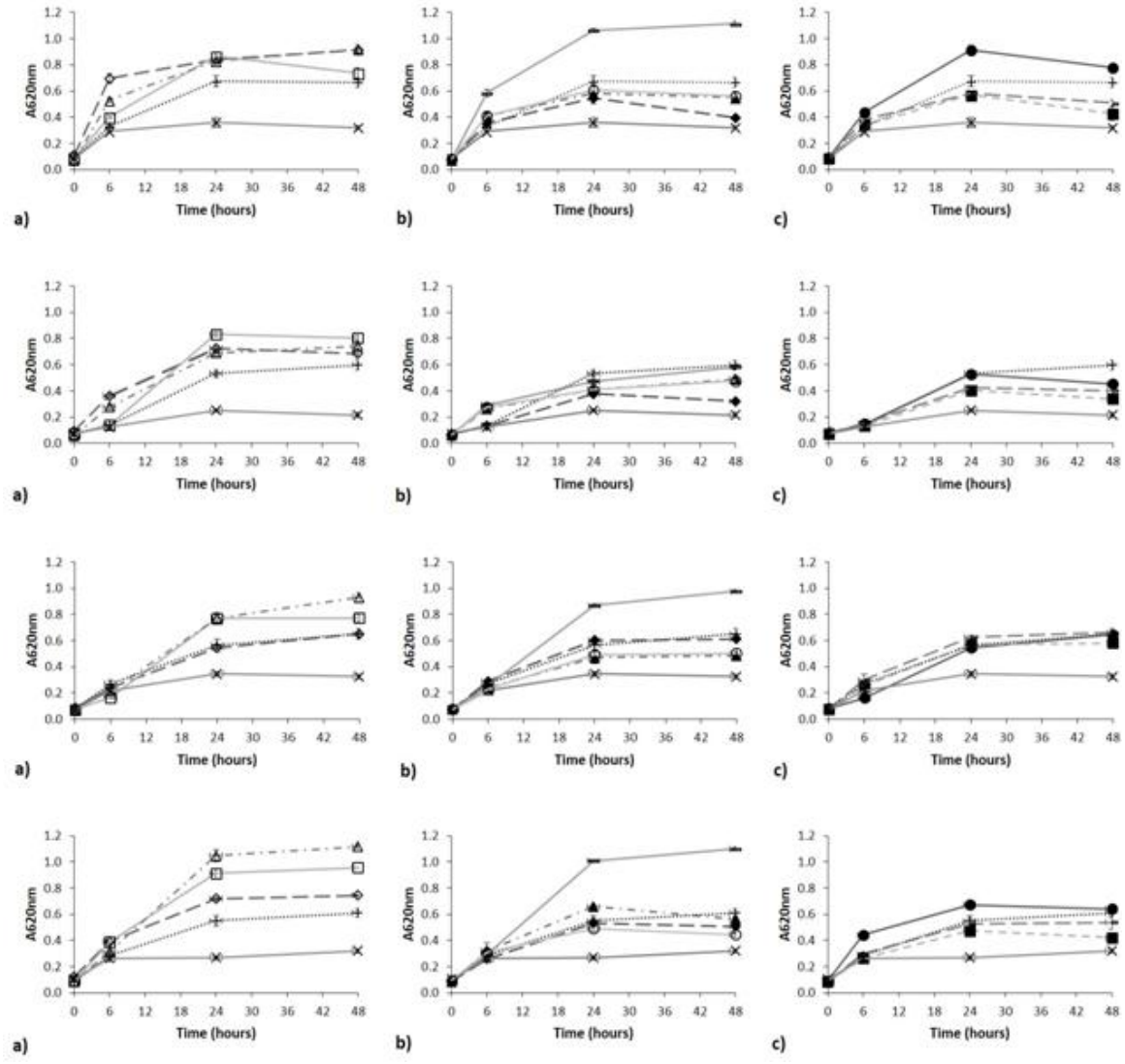

Figure 1: Growth curves of B632 (row 1), B1975 (row 2), B2274 (row 3), B7840 (row 4) using prebiotic fibers as sole carbon source: graphics a) = Vivinal® (◊), CUP-Oligo® (□), Actilight®950P (Δ); graphics b) = Frutafit® (♦), Orafti®HIS (-), Orafti®Sinergy (▲),Orafti®Raftiline HP (○); graphics c) = BioEcolians® (●), Arabinex® (■),Benefibra® (-). Glucose (+) and a solution without fiber, no fiber (×), were used as positive and negative control, respectively.

These tests highlighted different growth performances of the four *Bifidobacterium* strains. Generally, the sc-FOS Actilight®950P, together with the GOS CUP-oligo® assayed, were the substrates which best supported the growth of all the four strains. The GOS Vivinal® also showed good growth performances. *B. breve* B632 could grow very well on the oligofructose Orafti®HSI, with an increase in A_{620} of 1.12 ± 0.03 after 48 hrs of incubation. Orafti®HSI also supported the growth of *B. breve* B2274 and B7840 (Fig. 1), although to a less extent, whereas growth on this substrate is low for *B. longum* B1975 strain. On the other hand, Orafti®Synergy1 and Orafti®RaftilineHP sustained *Bifidobacterium* growth poorly, i.e. less than glucose. Similar results were obtained for Frutafit®, which could difficultly support growth of B632 and B1975 strain, whereas growth was comparable to that on glucose for the other two strains.

The third group of commercial fibers tested comprised polysaccharides traditionally not used in commercial products targeted to infants. These fibers were an α-glucooligosaccharide obtained enzymatically from maltose (BioEcolians®), a mixture of high MW arabinogalactans (Arabinex®) and a mixture of polysaccharides obtained from enzymatic hydrolysis of guar gum (Benefibra®). The *B. breve* B632 strain could grow well on these 3 fibers, in particular BioEcolians® sustained growth similarly to the FOS and GOS previously assayed (Fig. 2). The α-glucooligosaccharide was easily metabolized by the other *Bifidobacterium* strains, which, on the contrary, showed a reduced growth on other substrates.

The same fibers were tested as growth substrates on potential infant pathogens, i.e. a mixture of gas producing strains isolated from colicky infants (*E. coli, K. pneumonia* and *E. cloacae* in the ratio 1:1:1) and on a strain of *C. difficile*. The coliform mixture could grow well on glucose, but several differences were observed among the assayed oligosaccharides. CUP-oligo® stimulated the growth of the mixture, with an increase in A_{620} of 0.4 ± 0.04 at 48 h of incubation; Frutafit®, the α-glucooligosaccharide BioEcolians® and the partially hydrolyzed guar gum (Benefibra®) determined a growth increase of A_{620} of the enteric mixture of about 0.2, whereas, for all the other substrates, the increase of A_{620} was lower than 0.2 (data not shown). Growth curves were also obtained with *C. difficile* on the same polysaccharides. A significant increase of turbidity evaluated as A_{620} was observed after 48 h incubation with Benefibra®. Orafti®HSI, Frutafit®, BioEcolians® and Arabinex® showed a capability of sustaining *C. difficile* growth comparable to glucose (data not shown).

The prebiotic score was calculated for the assayed fibers. Using the coliform mixture as reference strains and averaging the scores obtained for the 4 *Bifidobacterium* strains, the oligofructose Orafty®HSI , the FOS Actilight®950P and the GOS Vivinal® obtained the highest prebiotic score, with mean values of 1.52, 1.39 and 1.18, respectively (Fig. 2A). In addition,

CUP-Oligo®, Orafti®Synergy, Orafti®Raftiline HP and BioEcolians® showed mean scores close to 1.00. Using *C. difficile* as reference strain, the highest prebiotic activity scores were obtained with Actilight®950P and Vivinal® (1.43 and 1.23 respectively) (Fig. 2B); on the contrary, Orafti®HSI showed a lower value (0.6) with respect to that obtained on the enteric mixtures. BioEcolians®, Frutafit® and Arabinex® showed negative values (-0.37, -0.55, -0.65, respectively), Benefibra® produced the lowest values (-1.67).

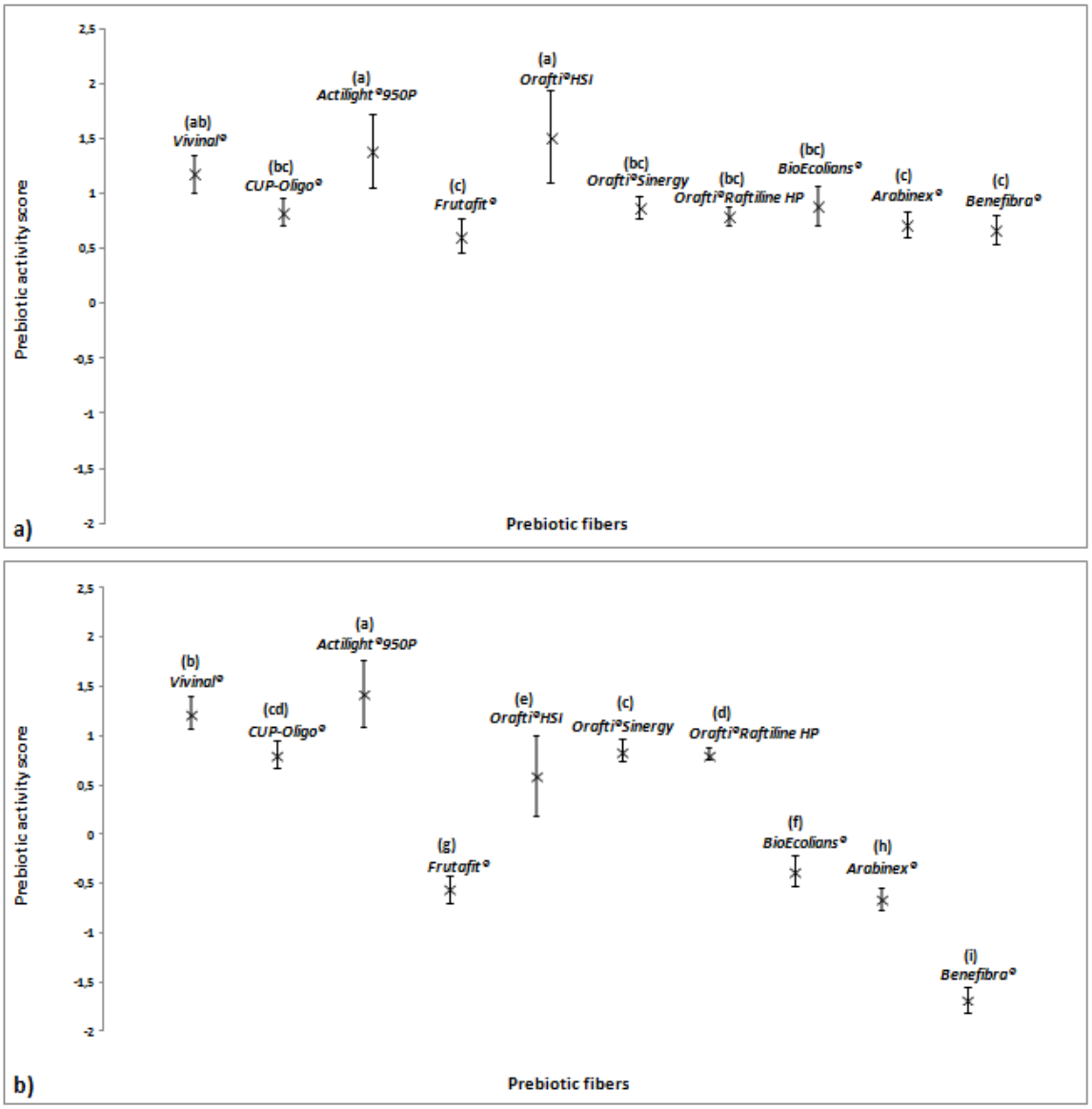

Figure 2: Prebiotic activity scores calculated by using the average of prebiotic scores obtained from each different *Bifidobacterium* strain (B632, B1975, B2274, B7840) considering the enteric mixture (*E. coli*, *K. pneumoniae*, *E. cloacae*, 1:1:1), (panel a) and *C. difficile*, (panel b), as target. Values are mean of four different replications ± standard deviations. Mean with different letters are significantly different at $P<0.05$.

Fig. 3A shows the prebiotic activity scores calculated for each *Bifidobacterium* strain, using the enteric mixture as the reference. A specific interaction between each strain and prebiotic fiber was shown. In particular the highest prebiotic activity scores were obtained with Orafti®HSI*B632, Orafti®HSI*B2274, Orafti®HSI*B7840 and Actilight®950P*B7840. Fig. 3B shows the prebiotic activity scores calculated for each single *Bifidobacterium* strain, using *C. difficile* as the reference. A specific strain-fiber interaction was observed, although to a less extent with respect to the use of the enteric mixture as the reference.

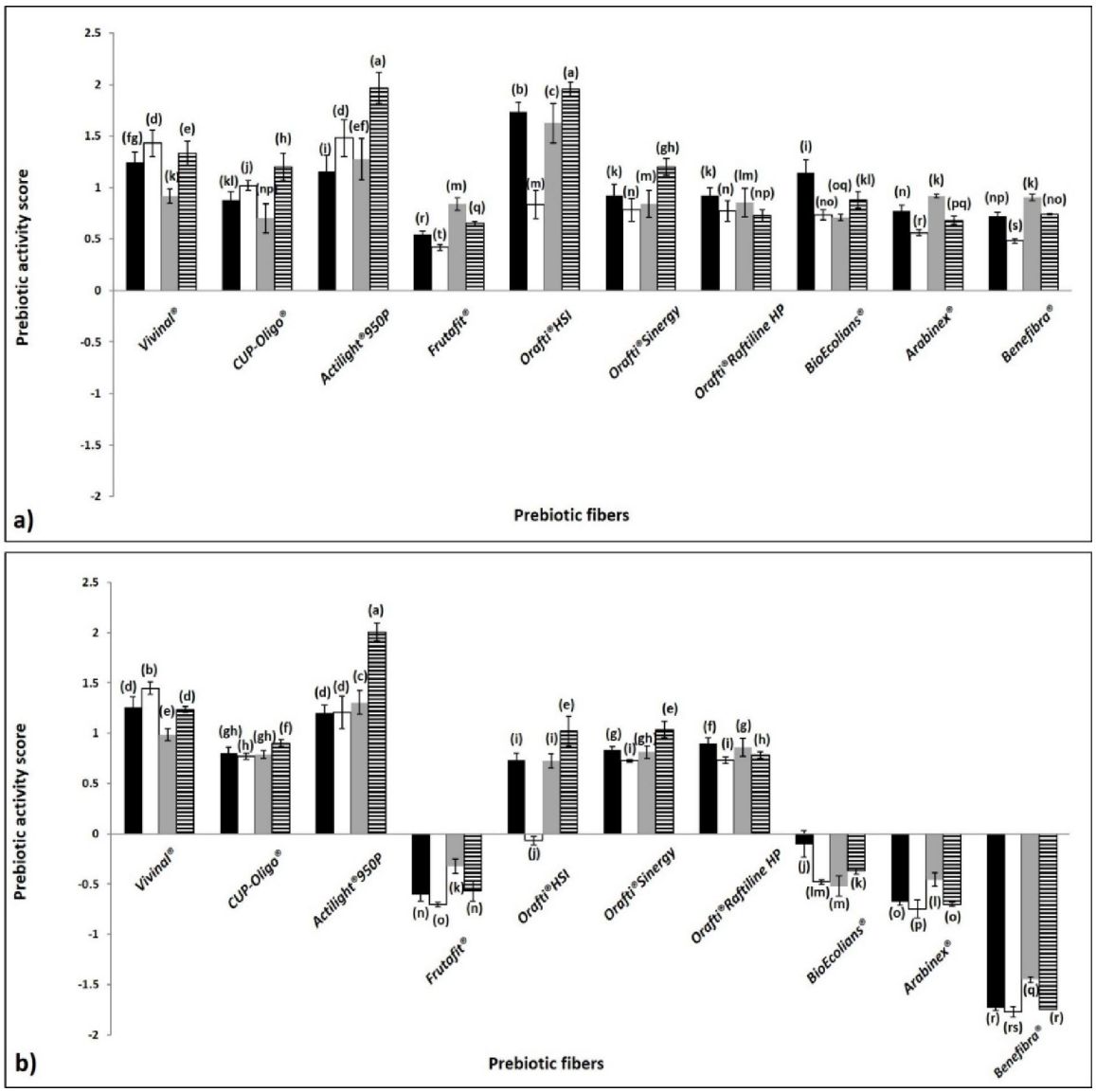

Figure 3: Prebiotic activity scores calculated for each *Bifidobacterium* strain (B632 = black column; B1975 = white column, B2274 = gray column, B7840 = striped column) considering the enteric mixture, (panel a), or *C. difficile*, (panel b), as target. Values are mean of three different replications ± standard deviations. Mean with different letters are significantly different at P<0.05.

3.2. Evaluation of strain survival under simulated intestinal conditions

Human gastric juices at pH 2.5 and 4 were used. A rapid decline in the cells counts was observed after 30 min at pH 2.5, corresponding to 4 Log reduction for B2274, 5 Log reduction for both B632 and B7840, and an almost complete reduction for B1975 (Table 2). No or negligible survival was detected after 60 min of incubation for all strains. When exposed to gastric juice at pH 4, the number of cells of *B. breve* B632 and B2274 did not decrease significantly after 30 min of incubation. On the contrary a reduction of one Log and three Log with respect to the initial concentration was detected for *B. breve* B7840 and *B. longum* B1975, respectively (Table 2). In addition, *B. breve* B632 exhibited an appreciable level of survival after 60 min of incubation. On the other hand, *B. longum* B1975 showed the worst survival, from 7.3 ± 0.03 Log CFU ml^{-1} to 4.0 ± 0.03 Log CFU ml^{-1} at the same incubation time. Resistance to low pH (gastric juice at pH 2.5) of microencapsulated *B. breve* B632 was also checked and compared to free cells. The survival of free and microencapsulated cells differed significantly: no death of the encapsulated cells at pH 2.5 was observed after 30 min exposure and less than 1.0 Log CFU ml^{-1} reduction was present after 60 min of exposure (Table 2).

As regards to the essay with bile salts, the reduction was statistically significant for all the strains, although *B. breve* B632 and B7840 showed only a 1.5 reduction after 1 h of exposure. *B. breve* B2274 showed a number reduction of 2.00 Log CFU ml^{-1} after 60 min of exposure and its viability decreased further after 120 min. *B. longum* B1975 showed the lowest resistance after 60 and 120 min of incubation (Table 2).

Table 2. Effect of human gastric juice and bile salts on survival *of Bifidobacterium* spp. selected strains. Results are shown as mean and standard deviation of experiment done in triplicate. Different letters (a-c) in the same row showed significant different at P<0.05. Different letters (A,B) in the same column showed significant difference at P<0.05.

	Survival count (Log10 CFU ml^{-1}) to human gastric juice at 2.5 pH			Survival count (Log10 CFU ml^{-1}) to human gastric juice at pH 4			Survival count (Log10 CFU ml^{-1}) to bile salts at pH 7		
Species/strain	**0 min**	**30 min**	**60 min**	**0 min**	**30 min**	**60 min**	**0 min**	**60 min**	**120 min**
B. breve B2274	8.6 ± 0.03 a	4.3 ± 0.24 b	2.3 ± 0.05 c	7.6 ± 0.11 a	7.5 ± 0.00 a	5.3 ± 0.28 b	8.1 ± 0.05 a	6.1 ± 0.26 b	5.5 ± 0.11 c
B. breve B7840	8.4 ± 0.21 a	2.7 ± 0.02 b	1.6 ± 0.43 c	7.7 ± 0.03 a	6.6 ± 0.01 b	5.0 ± 0.04 c	7.7 ± 0.00 a	6.4 ± 0.03 b	6.4 ± 0.00 b
B. longum B1975	8.0 ± 0.01 a	0.9 ± 0.00 b	0.5 ± 0.00 c	7.3 ± 0.03 a	4.4 ± 0.02b	4.0 ± 0.03 c	7.3 ± 0.56 a	5.7 ± 0.20 b	4.3 ± 0.10 c
B. breve B632	8.4 ±0.08aA	2.8 ± 0.05bB	0.2 ± 0.00cB	7.1 ± 0.71 a	7.0 ± 0.01 a	6.4 ± 0.11 b	8.5 ± 0.01 a	7.0 ± 0.06 b	6.4 ± 0.02 c
Microencapsulated B632	8.3 ±0.10aA	8.3 ± 0.03aA	7.4 ± 0.06bA	-	-	-	-	-	-

Discussion

The development of synbiotic products targeted to infants is important for the prevention or therapy of both gastrointestinal disorders and pathologies apparently not linked to gut health, such as allergies. This work was aimed at the evaluation of the prebiotic activity of several commercially available polysaccharides, including fibers usually employed in the human diet, such as FOS, GOS and inulin, but also less commonly used plant derived polysaccharides such as α-glucooligosaccharides, partially hydrolyzed guar gum and arabinogalactans, for their use in synbiotic products targeted to children. Strains previously selected for their interesting properties for infant use (*B. breve* B632, B2274, B7840 and *B. longum* B1975) were employed. The option of using these strains in a lyophilized or microencapsulated form was also evaluated in this work. Several studies have reported that the supplementation of infant formula with specific oligosaccharides, in particular GOS, stimulates the growth of bifidobacteria in the intestine resembling the effect of breast-feeding (Saavedra, 2007; Günther Boehm & Moro, 2008; G. T. Macfarlane, Steed, & Macfarlane, 2008). The study here described highlighted different growth performances of the assayed *Bifidobacterium* strains, in agreement with early studies reporting that carbohydrate utilization pattern differs greatly among *Bifidobacterium* species and, within each species, among different strains (Crociani, Alessandrini, Mucci, & Biavati, 1994). As a general trend, GOS, sc-FOS and oligofructose could sustain well the growth of the assayed strains, whereas higher MW FOS (inulins) were difficultly metabolized and stimulated the growth of only a restricted number of strains, the major effects being exerted on B632 and B7840. This specificity is in accordance with the observation that only a few *Bifidobacterium* strains produce extracellular hydrolytic enzymes necessary for FOS fermentation (Perrin, Warchol, Grill, & Schneider, 2001; Sims, Ryan, & Kim, 2014) and with the bifidobacteria general preference for the utilization of short chain oligofructose rather than long chain fructooligosaccharides such as high DP inulin (Rossi et al., 2005; Stewart, Timm, & Slavin, 2008). This explains why long chain polysaccharides such as Orafti®RaftilineHP (DP > 23) were difficultly fermented by the bifidobacteria tested in this work. However, a good prebiotic fiber should guarantee selective growth of beneficial bacteria in the colon, which means that it should not sustain growth of potentially harmful bacteria. In the case of newborns, gas producing coliforms are particularly detrimental because their carbohydrate fermentation can cause excessive intra-intestinal air production and pain in infants (Savino et al., 2011; Aloisio et al., 2012). In addition, *C. difficile* is a potential pathogen involved in infectious diarrhea in infants (Oğuz, Uysal, Daşdemir, Oskovi, & Vidinlisan, 2001). Moreover, a healthy gut microbiota in newborns is considered to be formed by the highest numbers of bifidobacteria and lowest

numbers of *C. difficile* and *E. coli* (Penders et al., 2006). The fibers showing the average highest prebiotic score referring to both a mixture of coliform bacteria and a strain of *C. difficile* were Orafti®HSI, Actilight®950P and Vivinal®. Benefibra®, Arabinex®, and Frutafit® showed the lowest prebiotic index. Regarding the prebiotic*strain interaction, the highest prebiotic scores were obtained with Orafti®HSI*B632, Orafti®HSI*B2274, Orafti®HSI*B7840 and Actilight®950P*B7840 when the enteric mixture is considered and Orafti®HSI*B2274, Vivinal®*B632, Vivinal®*B1975, Vivinal®*B7840. Therefore, a good synbiotic product for newborn use may be composed of one *Bifidobacterium* strains or a combination of them coupled to a fructooligosaccharide with a DP lower than 10, like Orafti®HSI or Actilight®950P and a GOS such as Vivinal®. Other oligosaccharides, such as the α-glucooligosaccharide Bioecolians®, although capable of sustaining growth of bifidobacteria, should not be considered for this purpose because of their stimulating activity on potential pathogenic strains. Moreover this results suggest to assay in further *in vivo* studies a mixture of GOS and FOS taking advantage of their synergic effect, as suggested and reported by several authors (G Boehm et al., 2002; Moro et al., 2006).

An essential feature for a probiotic strain is the ability to survive passage through the intestinal environment, reaching the colon in a lively state where it can multiply and exert beneficial effects (Guarner, 2006; Rijkers et al., 2010). The gastric juice is the strongest barrier for probiotics and, as suggested by Del Piano et al. (2011), the use of gastric juice *in vitro* is a reliable model to predict survival through gastric transit. The results obtained in this paper confirm that adult gastric pH is very hostile for bacterial survival. In newborns, following delivery, the gastric pH is close to neutrality (Bergman, 2013), it falls to 1.5-3 within a few hours but returns to neutrality in the following 24 hours (Morselli, Franco-Morselli, & Bossi, 1980; Bearer, 1995). pH subsequently declines very gradually, reaching adult values only after 2 years of age. Therefore, the resistance to gastric juice at pH 4, which is close to that of newborns and young infants (Kageyama, 2002), shown by some of the selected strains, such as *B. breve* B632 or B2274, is a positive feature for *Bifidobacterium* administration in a lyophilized form in young children. An alternative, which is unavoidable if the probiotic is targeted to children older than 1.5-2 years, is to administer strains coated in a gastro-resistant material which does not allow to have any losses upon gastric transit, as confirmed in our study. It is also significant to underline that microencapsulation of probiotic strains with a gastro-resistant coating should not only be regarded as a strategy to improve survival of strains after oral intake, but it is also a tool to improve shelf-life stability of the strains in different finished product matrices (Del Piano et al., 2011). The coating is hydrolyzed when pH raises again in the duodenum and is therefore very

important that free cells are able to survive in the presence of bile salts. Bile salts are released into the duodenum during digestion to solubilise fats coming from the diet and possess strong antimicrobial activity since they are able to disorganize the structure of the cell membrane as well as trigger DNA damage (Ruiz, Margolles, & Sánchez, 2013). Differently from gastric pH adaptation, which is very often negligible, bifidobacteria possess a variety of strain-specific bile resistance mechanisms which are responsible for adaptation to bile salts. As also described for other strains in the literature (Maragkoudakis, Chingwaru, Gradisnik, Tsakalidou, & Cencic, 2010; Santini et al., 2010; Ruiz et al., 2013), sensitivity to gastric pH or to bile salts is strain specific. Therefore, probiotic administration to newborns can be done using the strains in a lyophilized form, whereas, microencapsulation is necessary when gastric pH gets closer to that of adults.

Based on results obtained in this study, and also taking into consideration what obtained in our previous work (Aloisio et al., 2012), it is possible to consider the strain *B. breve* B632, as lyophilized strain or in a microencapsulated form, as a candidate microorganism for use in a synbiotic product targeted to infants coupled to a mixture of GOS and FOS (Vivinal® and Actilight®950P) or short chain oligofructose.

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References:

Aloisio, I., Santini, C., Biavati, B., Dinelli, G., Cencič, A., Chingwaru, W., … Di Gioia, D. (2012). Characterization of Bifidobacterium spp. strains for the treatment of enteric disorders in newborns. *Applied Microbiology and Biotechnology, 96*(6), 1561–76. doi:10.1007/s00253-012-4138-5

Bearer, C. F. (1995). How are children different from adults? *Environmental Health Perspectives, 103* Suppl , 7–12.

Bergman, N. J. (2013). Neonatal stomach volume and physiology suggest feeding at 1-h intervals. *Acta Paediatrica* (Oslo, Norway : 1992), *102*(8), 773–7. doi:10.1111/apa.12291

Bischoff, S. C. (2011). “Gut health”: a new objective in medicine? *BMC Medicine, 9*(1), 24. doi:10.1186/1741-7015-9-24

Boehm, G., Lidestri, M., Casetta, P., Jelinek, J., Negretti, F., Stahl, B., & Marini, a. (2002). Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. *Archives of Disease in Childhood. Fetal and Neonatal Edition, 86*(3), F178–81.

Boehm, G., & Moro, G. (2008). Structural and functional aspects of prebiotics used in infant nutrition. *The Journal of Nutrition, 138*(9), 1818S–1828S.

Crociani, F., Alessandrini, A., Mucci, M. M., & Biavati, B. (1994). Degradation of complex carbohydrates by *Bifidobacterium* spp. *International Journal of Food Microbiology, 24*(1-2), 199–210. doi:10.1016/0168-1605(94)90119-8

Del Piano, M., Carmagnola, S., Ballarè, M., Sartori, M., Orsello, M., Balzarini, M., … Capurso, L. (2011). Is microencapsulation the future of probiotic preparations? The increased efficacy of gastro-protected probiotics. *Gut Microbes, 2*(2), 120–123. doi:10.4161/gmic.2.2.15784

Di Gioia, D., Aloisio, I., Mazzola, G., & Biavati, B. (2014). Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Applied Microbiology and Biotechnology, 98*(2), 563–77. doi:10.1007/s00253-013-5405-9

Guarner, F. (2006). Enteric flora in health and disease. *Digestion, 73* Suppl 1, 5–12. doi:10.1159/000089775

Howard-Flanders, P., & Theriot, L. (1966). Mutants of *Escherichia coli* K-12 defective in DNA repair and in genetic recombination. *Genetics, 53*(6), 1137–50.

Huebner, J., Wehling, R. L., & Hutkins, R. W. (2007). Functional activity of commercial prebiotics. *International Dairy Journal, 17*(7), 770–775. doi:10.1016/j.idairyj.2006.10.006

Kageyama, T. (2002). Pepsinogens, progastricsins, and prochymosins: structure, function, evolution, and development. *Cellular and Molecular Life Sciences CMLS, 59*(2), 288–306. doi:10.1007/s00018-002-8423-9

Kumar, H., Salminen, S., Verhagen, H., Rowland, I., Heimbach, J., Bañares, S., … Lalonde, M. (2014). Novel probiotics and prebiotics: road to the market. *Current Opinion in Biotechnology, 32*C, 99–103. doi:10.1016/j.copbio.2014.11.021

Lian, W., Hsiao, H., & Chou, C. (2003). Viability of microencapsulated bifidobacteria in simulated gastric juice and bile solution. *International Journal of Food Microbiology, 86*, 293–301. doi:10.1016/S0168-1605(02)00563-9

Macfarlane, G. T., Steed, H., & Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology, 104*(2), 305–44. doi:10.1111/j.1365-2672.2007.03520.x

Maragkoudakis, P. a, Chingwaru, W., Gradisnik, L., Tsakalidou, E., & Cencic, A. (2010). Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection. *International Journal of Food Microbiology, 141* Suppl , S91–7. doi:10.1016/j.ijfoodmicro.2009.12.024

Marotti, I., Bregola, V., Aloisio, I., Di Gioia, D., Bosi, S., Di Silvestro, R., … Dinelli, G. (2012). Prebiotic effect of soluble fibres from modern and old durum-type wheat varieties on *Lactobacillus* and *Bifidobacterium* strains. *Journal of the Science of Food and Agriculture, 92*(10), 2133–40. doi:10.1002/jsfa.5597

Moro, G., Arslanoglu, S., Stahl, B., Jelinek, J., Wahn, U., & Boehm, G. (2006). A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Archives of Disease in Childhood, 91*(10), 814–9. doi:10.1136/adc.2006.098251

Morselli, P. L., Franco-Morselli, R., & Bossi, L. (n.d.). Clinical pharmacokinetics in newborns and infants. Age-related differences and therapeutic implications. *Clinical Pharmacokinetics, 5*(6), 485–527.

Mugambi, M. N., Young, T., & Blaauw, R. (2014). Application of evidence on probiotics, prebiotics and synbiotics by food industry: a descriptive study. *BMC Research Notes, 7*(1), 754. doi:10.1186/1756-0500-7-754

Oğuz, F., Uysal, G., Daşdemir, S., Oskovi, H., & Vidinlisan, S. (2001). The role of *Clostridium difficile* in childhood nosocomial diarrhea. *Scandinavian Journal of Infectious Diseases, 33*(10), 731–3.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., … Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics, 118*(2), 511–21. doi:10.1542/peds.2005-2824

Perrin, S., Warchol, M., Grill, J. P., & Schneider, F. (2001). Fermentations of fructo-oligosaccharides and their components by *Bifidobacterium infantis* ATCC 15697 on batch culture in semi-synthetic medium. *Journal of Applied Microbiology, 90*(6), 859–65.

Rijkers, G. T., Bengmark, S., Enck, P., Haller, D., Herz, U., Kalliomaki, M., … Antoine, J.-M. (2010). Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. *The Journal of Nutrition, 140*(3), 671S–6S. doi:10.3945/jn.109.113779

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., & Matteuzzi, D. (2005). Fermentation of Fructooligosaccharides and Inulin by Bifidobacteria : a Comparative Study of Pure and Fecal Cultures Fermentation of Fructooligosaccharides and Inulin by Bifidobacteria: a Comparative Study of Pure and Fecal Cultures. *Applied and Environmental Microbiology, 71*(10):6150-8. doi:10.1128/AEM.71.10.6150

Ruiz, L., Margolles, A., & Sánchez, B. (2013). Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. *Frontiers in Microbiology*, *4*(December), 396. doi:10.3389/fmicb.2013.00396

Saavedra, J. M. (2007). Use of Probiotics in Pediatrics: Rationale, Mechanisms of Action, and Practical Aspects. *Nutrition in Clinical Practice, 22*(3), 351–365. doi:10.1177/0115426507022003351

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., & Biavati, B. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *International Journal of Food Microbiology, 141* Suppl , S98–108. doi:10.1016/j.ijfoodmicro.2010.03.039

Savino, F., Cordisco, L., Tarasco, V., Locatelli, E., Di Gioia, D., Oggero, R., & Matteuzzi, D. (2011). Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. *BMC Microbiology, 11*, 157. doi:10.1186/1471-2180-11-157

Simone, M., Gozzoli, C., & Quartieri, A. (2014). The Probiotic *Bifidobacterium breve* B632 Inhibited the Growth of Enterobacteriaceae within Colicky Infant Microbiota Cultures. *BioMed Research …*, 2014.

Sims, I. M., Ryan, J. L. J., & Kim, S. H. (2014). In vitro fermentation of prebiotic oligosaccharides by *Bifidobacterium lactis* HN019 and *Lactobacillus* spp. *Anaerobe, 25*, 11–7. doi:10.1016/j.anaerobe.2013.11.001

Slavin, J. (2013). Fiber and prebiotics: mechanisms and health benefits. *Nutrients, 5*(4), 1417–35. doi:10.3390/nu5041417

Stewart, M. L., Timm, D. A., & Slavin, J. L. (2008). Fructooligosaccharides exhibit more rapid fermentation than long-chain inulin in an in vitro fermentation system. *Nutrition Research* (New York, N.Y.), *28*(5), 329–34. doi:10.1016/j.nutres.2008.02.014

Turroni, F., Peano, C., Pass, D. a, Foroni, E., Severgnini, M., Claesson, M. J., … Ventura, M. (2012). Diversity of bifidobacteria within the infant gut microbiota. *PloS One, 7*(5), e36957. doi:10.1371/journal.pone.0036957

PAPER 5

Influence of intrapartum antibiotic prophylaxis against group B *Streptococcus* on the early newborn gut composition and evaluation of the anti-*Streptococcus* activity of *Bifidobacterium* strains

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

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Abstract Several factors are known to influence the early colonization of the gut in newborns. Among them, the use of antibiotics on the mother during labor, referred to as intrapartum antibiotic prophylaxis (IAP), has scarcely been investigated, although this practice is routinely used in group B *Streptococcus* (GBS)-positive women. This work is therefore aimed at verifying whether IAP can influence the main microbial groups of the newborn gut microbiota at an early stage of microbial establishment. Fifty-two newborns were recruited: 26 born by mothers negative to GBS (control group) and 26 by mothers positive to GBS and subjected to IAP with ampicillin (IAP group). Selected microbial groups (*Lactobacillus* spp., *Bidobacterium* spp., *Bacteroides fragilis*, *Clostridium difficile*, and *Escherichia coli*) were quantified with real-time PCR on DNA extracted from newborn feces. Further analysis was performed within the *Bidobacterium* genus by using DGGE after amplification with genus-specific primers. Results obtained showed a significant decrease of the bifidobacteria counts after antibiotic treatment of the mother. Bifidobacteria were found to be affected by IAP not only quantitatively but also qualitatively. In fact, IAP determined a decrement in the frequency of *Bidobacterium breve, Bidobacterium bifidum*, and *Bidobacterium dentium* with respect to the control group. Moreover, this study has preliminarily evaluated that some bifidobacterial strains, previously selected for use in infants, have antibacterial properties against GBS and are therefore potential candidates for being applied as probiotics for the prevention of GBS infections.

Keywords Intrapartum antibiotic prophylaxis · Group B *Streptococcus* · *Bifidobacterium* spp · Probiotics · Antibacterial activity

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Introduction

It has been known for decades that a large number of commensal bacteria harbors in the gut, but only recent studies have begun to reveal the extraordinary complexity of the human microbiota (Cani and Delzenne 2011; Bäckhed et al. 2012; Grice and Segre 2012; Schloissnig et al. 2013). Such a complex microbial system is assembled soon after birth. The first microbial population the newborn comes in contact with is the maternal intestinal and vaginal microbiota; successively, the newborn is exposed to microbes from the environment. The first bacteria encountered in the majority of healthy infants are facultative anaerobes, then, with the reduction of the redox potential, strict anaerobes such as *Bifidobacterium* spp., *Bacteroides* spp., and *Clostridium* spp. become dominant (Solís et al. 2010; Sharon et al. 2013). However, it is well known that colonization in the early days after birth is influenced by several factors which were examined extensively within a large epidemiologic study (the KOALA study) carried out involving more than 1,000 newborns (Penders et al. 2006). The most important factors are: the mode of delivery, the maternal microbiota of intestine, vagina, and epidermis, the type of infant feeding (breast vs formula feeding), the use of antibiotics during the first few months of life, gestational age at birth, and hospitalization after birth. The KOALA study confirmed that exclusively breastfed vaginally born term

infants have the most healthy gut microbiota, with the highest numbers of bifidobacteria and lowest numbers of *Clostridium difficile* and *Escherichia coli.* Conversely, maternal lifestyle appears not to greatly influence gut microbial composition (Penders et al. 2006). The establishment of a proper commensal microbiota in the gut is crucial for the health of the newborn.

The primary cause of pathogen infection in vaginally delivered newborns is the maternal genital tract (Ferrieri and Wallen 2012). Early onset bacterial sepsis remains one of the major causes of neonatal morbidity and mortality (Ferrieri and Wallen 2012). The leading cause of infection in newborns is group B *Streptococcus* (GBS), mainly represented by *Streptococcus agalactiae* strains. This gram-positive bacterium resides in the cervix, vagina, or rectum and can reach the amniotic fluid through intact or ruptured membranes and lead to infection. It is estimated that about 10 % of pregnant women are positive to GBS (Al-Taiar et al. 2011). Sepsis-associated deaths have declined significantly in the last decade (2001–2011) due to the introduction of an intrapartum antibiotic prophylaxis (IAP) in GBS-positive women during labor (Puopolo et al. 2005; Ferrieri and Wallen 2012). Penicillin and ampicillin are used in IAP (Ferrieri and Wallen 2012). GBS is the causative agent of both early onset and late onset infection; in the first case, the infection manifests with respiratory disturbance and apneic episodes, while in the second case, with fever and poor feeding. As mentioned previously, there has been a significant decrease in the incidence of GBS infection to its current rate of approximately 0.32 per 1,000 live births for early onset disease, whereas there is no evidence that chemoprophylaxis prevents late onset disease. However, there is scarce information in the literature on the effect that the maternal antibiotic treatment may have on the early colonization of bacteria in the newborn gut, which is known to be highly influenced by the microorganisms that derive from the mother. A recent study (Keski-Nisula et al. 2013) has recorded a reduced vertical transmission of lactic acid bacteria from IAP-treated mothers to the neonates. However, analyses have been made with the use of cotton swabs taken both from the mother's vaginal tract and from the neonate's oral cavity and not by checking directly the newborn's feces.

Alternative therapies to the use of antibiotics for the prevention and treatment of GBS have not been considered up to now. Administration of probiotics during pregnancy has been evaluated in connection with the maintenance of a stable vaginal microbiota or with the reduction of eczema (Wickens et al. 2008) and atopic dermatitis (Dotterud et al. 2010) in early infancy. However, to the best of our knowledge, only one study has considered the antimicrobial property against GBS of some *Lactobacillus* strains (Zárate and Nader-Macias 2006) for their use as potential probiotics in GBS-positive pregnant women.

This work is aimed at the evaluation of the influence that maternal antibiotic profilaxis against GBS may have on the main microbial groups present in the newborn gut microbiota. Moreover, the antimicrobial activity of *Bifidobacterium* spp. strains, previously selected as potential probiotic strains with no harmful effects on newborns (Aloisio et al. 2012), against GBS has been evaluated with the idea of using them as probiotics in pregnant women to prevent infection of GBS.

Materials and methods

Study design and sample collections

The study was performed on 52 newborns enrolled by the Neonatal Intensive Care Unit of the S. Orsola-Malpighi Hospital of Bologna from April 2013 to December 2013 (Table 1). Inclusion criteria were: born at term by vaginal delivery, birth weight adequate for gestational age (2.5–4.0 kg), exclusively breastfed (in order to reduce variability in the intestinal microbiota consequent to diet), not receiving perinatal antibiotic treatment, and not receiving perinatal probiotic treatment. Fecal samples were collected from recruited newborns at 6th–7th days after birth. Twenty-six infants were born by mothers resulted negative to group B *Streptococcus* (GBS) after vaginal swab (control group) and 26 infants by mothers positive to GBS and treated with 2 g of ampicillin (Amplital®) at least 4 h before delivery, followed eventually by 1 g every 4 h until delivery (IAP group). Administration was performed in agreement with the international guidelines of the Center of Disease Control and Prevention (Puopolo et al. 2005). In the period in which the study has been performed, only two mothers received a different antibiotic treatment because of allergy to ampicillin. They have been excluded from the trial in order to reduce the variability due to different types of antibiotics.

The study was approved by the local ethics committee (*Comitato Etico Indipendente dell'Azienda Ospedaliero-Universitaria di Bologna*, Policlinico S. Orsola-Malpighi, document number 12/2013/U/Oss approved on March 12, 2013) before it started.

Fecal samples were collected during the medical examination at 7 days after birth and they were immediately frozen at −80 °C, in numbered screw-capped plastic containers, until they were processed for DNA extraction. Researchers carrying out DNA extraction and microbial analyses were blind to the group identity of infants (control group or IAP group).

DNA extraction from fecal sample

Two hundred milligrams of newborn feces (preserved at −80 °C after collection) were used for the DNA extraction using the QIAamp DNA Stool Mini Kit (QIAGEN, West

Table 1 Sample origin and demographic characteristics

Sample	Sampling age (days)	Nutrition	Delivery mode	Gender		Nationality	
				Female	Male	Italian	Not Italian
Control group	7	Breast fed	Vaginal	13	13	22	4
IAP group	7	Breast fed	Vaginal	14	12	25	1

Sussex, UK) with a slight modification: an additional incubation at 95 °C for 10 min of the stool sample with the lysis buffer was added to the standard protocol to improve the bacterial cell rupture. Extracted DNA was stored at −80 °C. The purity and concentration of extracted DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland).

Quantitative PCR

Quantification of selected microbial groups of the newborn gut microbiota (*Lactobacillus* spp., *Bidobacterium* spp., *Bacteroides fragilis* group, *C. difficile* and *E. coli*) was carried out with real-time PCR on DNA extracted from fecal 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 (Tables 2 and 3), H_2O molecular grade and 2-μl DNA extracted from fecal samples at a concentration of 2.5 ng/μl for all the assays except *C. difficile* quantification. For *C. difficile* quantification, DNA extracted from fecal samples was not diluted (15–25 ng/μl). 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 (Altschul et al. 1990). 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 cells per gram of feces, expressed as log colony forming unit (CFU)/g, according to the ribosomal RNA (rRNA) copy number available at the rRNA copy number database (Klappenbach et al. 2001; Lee et al. 2009). Equations and coefficients of determination for the different assays are reported in Table 2.

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, and 10^7 copies of the gene per reaction were used for calibration.

Data of microbial counts were subjected to one-way analysis of variance (ANOVA) in order to evidence significant differences between treated and control group of newborns.

Denaturing gradient gel electrophoresis analysis

Bifidobacterium genus-specific PCR primers targeted on the 16S rRNA gene were used in this study. They were Bif164-F (GGGTGGTAATGCCGGATG) and Bif662-R (CCACCGTT ACACCGGGAA). A 40-bp GC clamp (CGCCCGCCGCGC GCGGCGGGCGGGGCGGGGGCACGGGGGG) has been attached to the 5′ end of Bif662-R as described by Satokari et al. (2001). These primers were provided from MWG Biotech (Ebersberg, Germany). PCR reactions were basically performed as described by Satokari et al. (2001), using the HotStarTaq polymerase kit from QIAGEN. Each PCR mixture (30 μl) contained 1×PCR buffer (QIAGEN), 3-mM MgCl2, 0.2 mM of each dNTP, 0.2 mM of each primer, 1.25-U Taq polymerase, and 3 μl of appropriately diluted template DNA (15 ng/μl). The PCR thermocycling program was the following: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 40 s; and final extension at 72 °C for 7 min. The reactions were subsequently cooled to 4 °C. PCR amplicons were estimated by analyzing 5-μl samples in a 1.3 % agarose gel (*w/v*) electrophoresis and staining with ethidium bromide. PCR amplification products were stored at −20 °C until denaturing gradient gel electrophoresis (DGGE) analysis was carried out with a D-Code electrophoresis system (Bio-Rad Labs, Hercules, CA). DGGE gels were performed with 7 % (*w/v*) polyacrylamide (37.5:1 acrylamide-bisacrylamide) in 1X Tris–acetate–EDTA (TAE) buffer according to Muyzer and Smalla (1998). A denaturing gradient of 50 to 55 % urea–formamide (100 % corresponding to 7-M urea and 40 %*v/v* formamide) was applied for the separation of the amplicons. Electrophoretic runs were at 150 V for 10 min and then at a constant voltage of 55 V and a temperature of 60 °C for 16 h. DNA isolated from species most commonly present in newborn fecal samples, i.e., the reference strains *Bifidobacterium breve* DSM 20213^T, *Bifidobacterium bifidum* DSM 20452^T, *Bifidobacterium longum* subsp. *infantis* DSM 20088^T, *Bifidobacterium longum* subsp. *longum* DSM 20219^T, and *Bifidobacterium pseudocatenulatum* DSM 20438^T and amplified in the same

Table 2 Primer sequences and qPCR equations used in the different assays

Target microorganisms	Primer sequences (5′–3′)	Amplicon length (bp)	References	Equation	R^2
Escherichia coli					
Eco-F	GTTAATACCTTTGCTCATTGA	340	Malinen et al. 2003	Ct=−3.63x+38.50	0.998
Eco-R	ACCAGGGTATCTAATCCTGTT				
Clostridium difficile					
Cdiff-F	TTGAGCGATTTACTTCGGTAAAGA	114	Penders et al. 2006	Ct=−3.58x+39.61	0.998
Cdiff-R	TGTACTGGCTCACCTTTGATATTCA				
Bifidobacterium spp.					
BiTOT-F	TCGCGTCYGGTGTGAAAG	243	Rinttilä et al. 2004	Ct=−3.38x+41.06	0.995
BiTOT-R	CCACATCCAGCRTCCAC				
Lactobacillus spp.					
Lac-F	GCAGCAGTAGGGAATCTTCCA	349	Castillo et al. 20066	Ct=−3.84x+34.93	0.999
Lac-R	GCATTYCACCGCTACACATG				
Bacteroides fragilis group					
Bfra-F	CGGAGGATCCGAGCGTTA	92	Penders et al. 2006	Ct=−3.34x+40.48	0.989
Bfra-R	CCGCAAACTTTCACAACTGACTTA				

way fecal DNA was used to prepare a reference ladder. Gels were stained with SYBR Safe (Life Technologies Italia, Monza, Italy) for 20 min, rinsed with 1X TAE buffer and then displayed under UV light. Digital capturing was performed by using a Gel Doc™ XR apparatus (Bio-Rad). The most interesting bands were cut with a sterile scalpel from the denaturing gels, eluted in sterile water at 4 °C, re-amplified and re-analyzed in DGGE. The eluted bands were then used as a template to re-amplify the band fragments using the same primers without the GC clamp. The PCR products were purified from agarose gel using the NucleoSpin® Extract II kit (MACHEREY-NAGEL GmbH & Co. KG) and sequenced (Eurofins MWG Operon, Ebersberg, Germany). Sequence chromatograms were edited and analyzed using the software programs Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and obtained sequences were analyzed using the BLAST program.

Experimental design and statistical analysis

All the tests performed with real-time PCR assays were performed in triplicate and microbial counts obtained were subjected to one-way analysis of variance (ANOVA) by using the Statistica Software (ver. 7.1 StatSoft, Tulsa, Oklahoma, USA). The ANOVA test was carried out in order to evidence significant differences between treated and control samples.

The correspondence analysis (CA) was applied to the fingerprinting pattern obtained from PCR-DGGE analysis of bifidobacterial population of newborn fecal samples. CA is a statistical method for visualizing the association between levels of a two-way contingency table (Benzecri 1992). Banding profiles were scored as the presence/absence of different *Bifidobacterium* species in each investigated sample. The contingency table was analyzed by CA module Statistica Software (ver. 7.1, StatSoft, Tulsa, OK, USA). Plotting the

Table 3 qPCR cycles and primer concentrations using SybrGreen chemistry for the different assays

Taget bacteria	Initial denaturation	Denaturation	Annealing temperature	N cycles	Fw (nM)	Rev (nM)
E. coli						
Eco-F/Eco-R	95 °C - 20 s	95 °C - 3 s	60 °C - 30 s	40	400	400
C. difficile						
Cdiff-F/Cdiff-R	95 °C - 20 s	95 °C - 3 s	60 °C - 30 s	40	250	250
Bifidobacterium spp.						
BifTOT-F/BifTOT-R	95 °C - 20 s	95 °C - 3 s	60 °C - 35 s	40	200	300
Lactobacillus spp.						
Lac-F/Lac-R	95 °C - 20 s	95 °C - 3 s	63.5 °C - 30 s	40	200	200
B. fragilis group						
Bfra-F/Bfra-R	95 °C - 20 s	95 °C - 3 s	60 °C - 30 s	40	300	300

first two dimensions of the coordinates of cases (frequencies of bifidobacterial species obtained by PCR-DGGE profiles) and variables (newborn fecal samples belonging to two groups: IAP and control group) gave a view of correspondence among investigated newborns, *Bifidobacterium* species, and IAP treatment. The first and second dimensions explained 19.37 and 16.67 % of total variability, respectively.

Antimicrobial activity of *Bifidobacterium* spp. strains against *Streptococcus agalactiae* using agar spot test

Four strains of *Bifidobacterium*, i.e., *Bifidobacterium breve* B632 (DSM 24706), *Bifidobacterium breve* B2274 (DSM 24707), *Bifidobacterium breve* B7840 (DSM 24708), and *Bifidobacterium longum* subsp. *longum* B1975 (DSM 24709), previously characterized in Aloisio et al. (2012), were used to evaluate their potential antimicrobial activity against *S. agalactiae* strains. *Bifidobacterium* strains were cultivated in tryptone, peptone, and yeast (TPY) extract medium (Santini et al. 2010) and incubated at 37 °C under anaerobic conditions using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany). Four *S. agalactiae* strains were used in this assay: *S. agalactiae* DSM 2134^T and three strains isolated from vaginal swabs of GBS-positive pregnant women and kindly supplied by the Microbiology unit of the S. Orsola Malpighi Hospital of Bologna. Identification of the isolated *S. agalactiae* strains was performed according to Phillips et al. (1980).

The basic protocol of the spot agar test employing whole cells has been described in Santini et al. (2010). Briefly, 10 μl of each *Bifidobacterium* strain exponentially grown culture, having an absorbance at 600 nm (A_{600}) of approximately 0.8–1, were spotted on TPY agar plates which were then incubated in anaerobic conditions for 24 h at 37 °C. Plates were then overlaid with 10 ml of brain heart infusion (BHI, Oxoid, Basingstoke, UK) broth, added with 0.7 % agar, containing 500-μl of the *S. agalactiae* cell suspension (A_{600} of 0.1). After drying, plates were incubated for 24 h in aerobic conditions and the growth inhibition halos were evaluated and measured. Five microliters of acetic acid (1 M) was used as a positive control and sterile BHI broth was used as a negative control. Each assay was performed in triplicate.

In addition to the spot agar text with live cells, inhibition assays were performed using the cell culture supernatants, both neutralized (i.e., brought to pH 7) and non-neutralized (i.e., with no pH correction, pH in the range of 4.5–5.5). Culture supernatants were prepared after centrifugation at 15,000×*g* for 20 min at 4 °C of bifidobacteria o.n. cultures followed by filtration through a 0.22-μm pore size cellulose acetate filter. The procedure described in Savino et al. (2011) was used. Briefly, nutrient agar plates (1.5 % agar, Oxoid) were prepared, cooled to about 40 °C and inoculated with 500 μl of *S. agalactiae* culture at the concentration of 10^7 CFU per milliliter. Fifty microliters of neutralized and non-neutralized culture supernatant were used to imbibe sterile paper blank disks (diameter 6 mm) which were placed on the agar plates. After 48 h of incubation at 37 °C, the inhibition zones were observed. The experiments were made in triplicate.

Results

Quantification of selected microbial groups in newborn fecal samples

DNA was extracted from fecal samples obtained from 52 newborns aged 6–7 days: 26 born from mothers treated intrapartum with ampicillin and 26 controls. Quantification of selected microbial groups of the newborn gut microbiota (*Lactobacillus* spp., *Bidobacterium* spp., *Bacteroides fragilis* group, *C. difficile*, and *E. coli*) was carried out with real-time PCR. The average microbial counts obtained are shown in Table 4.

All the microbial groups assayed were detected in all fecal samples, although the average quantitative counts differed greatly among each microbial group. In the samples belonging to the control group, *E. coli*, *Bacteroides fragilis* group, and bifidobacteria were the most abundant (9.03, 8.53, and 7.29 log CFU/g, respectively), whereas the counts of lactobacilli and *C. difficile* were much lower (6.73 and 3.70 log CFU/g, respectively). A great variability of the microbial counts among the different newborns was also observed: bifidobacteria were the group with the largest range of counts (the difference between the highest and the lowest counts was 6.83 log CFU/g). In the group whose mothers received IAP, a significant average reduction of the number of *Bifidobacterium* spp. (from an average of 7.29 to 5.85 log CFU/g) was observed (Table 3). In addition, the difference between the highest and the lowest counts obtained within the newborns was reduced with respect to the control group (4.55 log CFU/g). A reduction of 1 log of the *E. coli* count was found in the stools of newborns born from treated women with respect to control samples, although these data did not result significant probably because of the wide variability within each group of samples. The other microbial genera and species analyzed (*Lactobacillus* spp., *C. difficile*, and *Bacteroides fragilis*) were not significantly affected by the maternal treatment with ampicillin. *Lactobacillus* spp. and *C. difficile* counts do not show great variability within and between the two groups. *Bacteroides fragilis* group counts found in the two groups showed a similar average value (8.52 log CFU/g and 9.16 log CFU/g) with a great variability within each group.

Table 4 Mean counts of different microbial groups analyzed in newborn stool sample expressed as log (CFU/g of feces)

Target	Log CFU/g of feces in the following microbial groups				*p* value
	Control group (*n*=26)		IAP group (*n*=26)		
	Mean	Range	Mean	Range	
Bifidobacterium spp	7.29	4.12–10.95	5.85	3.24–7.79	0.001*
Lactobacillus spp	6.73	5.45–8.20	6.69	5.40–8.93	NS
E. coli	9.03	5.61–11.78	8.18	4.09–12.70	NS
C. difficile	3.70	2.85–5.46	3.89	3.12–4.80	NS
B. fragilis group	8.53	5.22–11.16	8.17	4.68–11.99	NS

NS not significant

DGGE analysis with *Bifidobacterium* genus-specific primers

To better characterize the changes in the bifidobacterial population that were observed with real-time PCR, PCR-DGGE analyses using genus-specific primers targeted to bifidobacteria were carried out both on the IAP samples and on the controls. Profiles analysis of all the samples showed a lower level of diversity of IAP group samples with respect to control group samples. Figure 1 shows an exemplificative profile of six IAP and six control samples. The six profiles corresponding to IAP group samples showed a reduced number of bands with respect to the profiles of control group samples. Most of the bands could be identified by comparing the migrant distances of their respective PCR amplicons with those of reference strains used as markers (e.g., the highest band in the profile could be ascribed to *Bifidobacterium breve*; bands 4, 5, and 6 to *Bifidobacterium bifidum*, band 7 to *Bifidobacterium longum* and band 14 to *Bifidobacterium pseudocatenulatum*). *Bifidobacterium longum* subsp. *infantis* DSM 20088^T and *Bifidobacterium longum* subsp. *longum* DSM 20219^T could not be separated with the DGGE gradient used. Identity was confirmed by cutting and sequencing of some of the bands (Table 5). A number of bands were not present in the mixture of type strains used and were identified only by sequencing (e.g., *Bifidobacterium dentium* and *Bifidobacterium pseudolongum* subsp. *pseudolongum*; the latter is not present in the DGGE profile presented in Fig. 1). In addition, some *Bifidobacterium breve* fragments of the fecal samples migrated to a different position than those of the culture collection strains (e.g., bands 9, 10, and 11), as already evidenced for some *Bifidobacterium* species in the work of Satokari et al. (2001) and were identified by sequencing. Sequencing results and GenBank accession numbers are shown in Table 5. Frequency values of the most abundant *Bifidobacterium* species were calculated for all the 52 samples (26 IAP groups and 26 control group samples) and shown in a radar chart (Fig. 2). IAP determined a strong decrement in the frequency of *Bifidobacterium breve* (50 % control group vs 25 % IAP group), *Bifidobacterium bifidum* (50 % control group vs 25 % IAP group), and *Bifidobacterium dentium* (38 % control group vs 13 % IAP group). On the other hand, *Bifidobacterium pseudocatenulatum* (13 % control group vs 13 % IAP group), *Bifidobacterium pseudolongum* (56 % control group vs 50 % IAP group), and *Bifidobacterium longum* (81 % control group vs 81 % IAP group) seemed to be less influenced by the treatment.

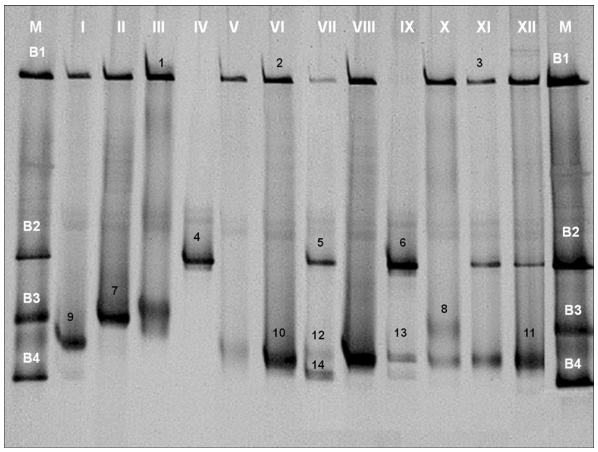

Fig. 1 DGGE of PCR products of 12 newborn fecal samples after amplification with *Bifidobacterium* genus-specific primers: IAP group samples (lanes I to VI), control group samples (lanes VII to XII) and mixed PCR products from pure culture (lanes M). *lane M*: *B1 B. breve* DSM 20213^T; *B2 B. bifidum* DSM 20452^T; *B3 B. longum* subsp. *infantis* DSM 20088^T and *B. longum* subsp. *longum* DSM 20219^T; *B4 B. pseudocatenulatum* DSM 20438^T. *Numbers* indicate bands that were cut and sequenced

Furthermore, a correspondence analysis (CA) was carried out (Fig. 3). The CA and scatterplot projections of variables (*Bifidobacterium* species frequencies) and cases (IAP group and control group newborn samples) on the first two dimensions evidenced one cluster formed by *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium longum* associated with IAP group samples. On the opposite part of the first axis, there is a second main cluster composed by *Bifidobacterium breve, Bifidobacterium bifidum*, and *Bifidobacterium dentium* associated with the

Table 5 Best-mach identification phylotypes of excised DGGE bands amplified with primers targeted on *Bifidobacterium* 16S rDNA gene

Bands	GenBank accession number	Closest match (NCBI accession number)	Percentage of identity (%)
1	KF990563	*Bifidobacterium breve* (NC020517.1)	100
2	KF990565	*Bifidobacterium breve* (NC020517.1)	100
3	KF990562	*Bifidobacterium breve* (NC020517.1)	100
4	KF990559	*Bifidobacterium bifidum* (NC017999.1)	99
5	KF990560	*Bifidobacterium bifidum* (NC017999.1)	99
6	KF990561	*Bifidobacterium bifidum* (NC017999.1)	99
7	KF990570	*Bifidobacterium longum* (NC0210008.1)	99
8	KF990571	*Bifidobacterium longum* (NC021008.1)	99
9	KF990564	*Bifidobacterium breve* (NC020517.1)	99
10	KF990566	*Bifidobacterium breve* (NC020517.1)	99
11	KF990567	*Bifidobacterium breve* (NC020517.1)	99
12	KF990568	*Bifidobacterium dentium* (NC013714.1)	99
13	KF990569	*Bifidobacterium dentium* (NC013714.1)	99
14	KF990572	*Bifidobacterium pseudocatenulatum* (NC037117.1)	99

control group samples. This further evaluation confirmed the previous data assessments.

Antimicrobial activity against *S. agalactiae* strains

The antimicrobial activity with the spot agar test employing whole cells was evaluated measuring the *radius* of the halo that surrounds the *Bifidobacterium* spot. The results obtained with the four *Bifidobacterium* strains (*Bifidobacterium breve* B632, B2274, and B7840 and *Bifidobacterium longum* subsp. *longum* B1975) evidenced a marked antimicrobial activity against all four *S. agalactiae* strains. An example of the halos obtained with the *S. agalactiae*-type strain is shown in Fig. 4.

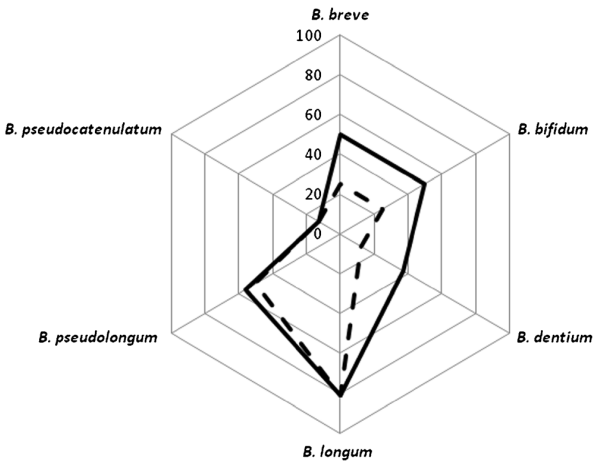

Fig. 2 Radar chart displaying the frequencies values of each *Bifidobacterium* species obtained by DGGE technique. The six axes correspond to each single *Bifidobacterium* species frequency (% values), the area surrounded by continuous line corresponds to the IAP group of newborn and the area surrounded the *dotted line* indicates the control group of newborn

The halo's *radius* was similar for the three *Bifidobacterium breve* strains, being higher than 1 cm, whereas *Bifidobacterium longum* subsp. *longum* B1975 strain showed inhibition halo's *radius* lower than 0.5 cm against all *S. agalactiae* strains. Inhibition halos were also obtained by using culture supernatants with the assay using imbibed paper disks; halos had the same extent of those obtained with whole cells (data not shown). On the contrary, with the use of neutralized culture, supernatant halos were only obtained with *Bifidobacterium breve* B632.

Discussion

Microbiological research abundantly focused on the gut microbiota in early infancy and on the independent effect of

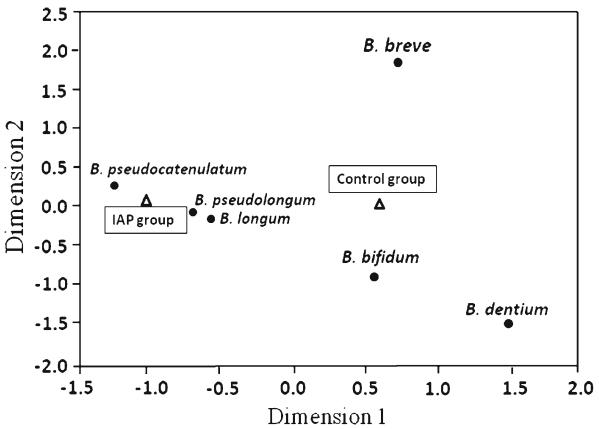

Fig. 3 Biplot of the relation established between frequencies values of the *Bifidobacterium* species (*solid circles*) after elaboration of the DGGE band patterns and the two groups of newborns (IAP group and control group, *empty squares*) obtained by correspondence analysis (CA)

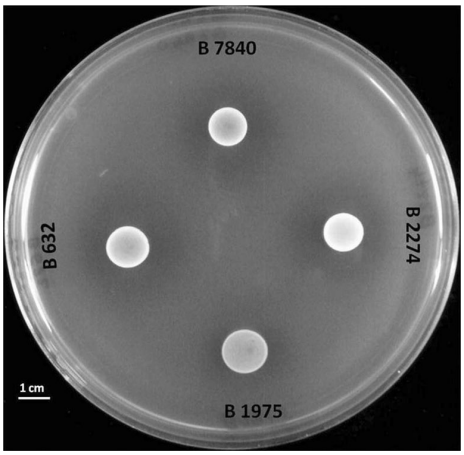

Fig. 4 Spot agar test evidencing the antimicrobial activity of four *Bifidobacterium* strains (*B. breve* B632, *B. breve* B2274, *B. breve* B7840, and *B. longum* subsp. *longum* B1975) against *S. agalactiae* DSM 2134^T. Inhibition halos were obtained with spot agar test using TYP agar plates

different factors in shaping microbial composition (Penders et al. 2006; O'Sullivan et al. 2013). Conversely, the possible effects on the newborn microbiota of IAP against GBS have been scarcely investigated, although this practice is routinely used in Europe and USA. This work is therefore aimed at verifying whether IAP can have an influence on the main microbial groups of the newborn gut microbiota at an early stage. In addition, the antimicrobial activity of *Bifidobacterium* spp. strains against GBS has been preliminarily explored with the perspective of using them as probiotics in pregnant women to reduce or prevent GBS infection.

Real-time PCR was used for quantitative analyses of the main microbial groups present in newborn fecal samples, as already done in other studies focusing on factors affecting neonate microbial composition (Palmer et al. 2007). The results obtained confirmed the great variability in the newborns' microbial composition (Palmer et al. 2007; Sanders et al. 2010). Intra-group variation mainly regards the counts of *E. coli*, *Bacteroides fragilis* group, and *Bifidobacterium* spp. However, the main achievement of this work is the significant reduction in the bifidobacteria counts following IAP. It is known that oral use of beta-lactam antibiotics in neonates in the first month of life results in decreased numbers of bifidobacteria (Penders et al. 2006; Mangin et al. 2010). This reduction is also present in neonates born by natural delivery when ampicillin is administered intrapartum to the mother. A previous work on the effect of IAP on lactic acid bacteria reported a decreased transmission rates of these microorganisms to the neonates evaluated with the use of an oral swab in the newborn (Keski-Nisula et al. 2013). The results obtained within our work show that maternal IAP does not influence the amount of lactic acid bacteria in the neonate intestine.

Bifidobacteria were found to be affected by IAP not only quantitatively but also qualitatively, as evidenced by PCR-DGGE analyses. It is well known that bifidobacterial composition in infants is usually less stable compared to adults (Satokari et al. 2002) and that several factors may influence the species distribution and abundance (Penders et al. 2006; Sanz et al. 2007; Fuligni et al. 2012; Di Gioia et al 2014). The diversity of bifidobacteria population in 11 newborn fecal samples has recently been investigated using pyrosequencing (Turroni et al. 2012). According to these authors, the dominant bifidobacterial species were *Bifidobacterium longum* followed by *Bifidobacterium bifidum* and *Bifidobacterium breve*. Our results on the control group confirmed the prevalence of these species, in addition to *Bifidobacterium pseudolongum*. Conversely, IAP determined a strong decrement in the frequency of *Bifidobacterium breve, Bifidobacterium bifidum*, and *B. dentium*, therefore confirming the important effect that IAP has on the early bifidobacterial colonization. This is further evidenced by the results of the CA which showed one cluster formed by *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium longum* associated with IAP group samples and another cluster composed by *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium dentium* associated with the control group.

Considering the high variability in antibiotic sensitivity among bifidobacteria species and strains (D'Aimmo et al. 2007; Mättö et al. 2007; Ammor et al. 2008), it is extremely difficult to find an antibiotic that can be active against GBS without affecting bifidobacteria. Moreover, it is well known that the large use of antibiotics can help the spreading of antibiotic resistances among bacteria (Bush et al. 2011). An increase in ampicillin-resistant *E. coli* strains was detected in newborns following IAP with this antibiotic (Bizzarro et al. 2008). Therefore, alternatives to traditional chemotherapy are looked for. Several works confirm the efficacy of probiotics for the prevention and treatment of several pathologies including vaginal infections (Chiang and Pan 2012; Amaretti et al. 2013), but only a few studies has been focused on *S. agalactiae* infection in pregnant women (Zárate and Nader-Macias 2006). In the present work, evidences are presented showing that some bifidobacterial strains, previously selected for use in infants and therefore safe for the newborn (Aloisio et al. 2012), posses in vitro antibacterial properties against *S. agalactiae* strains. They have, therefore, the potential for being used as probiotics for the prevention of *S. agalactiae* infections. Further studies are necessary to understand the mechanisms of the antibacterial activity of the bifidobacteria strains against the urinary pathogen considered in this work. This is particularly interesting for *Bifidobacterium breve* B632 strain whose neutralized culture

supernatant shows antibacterial activity against *S. agalactiae* (this work) and against other pathogenic strains (Aloisio et al. 2012). For this strain, the inhibitory activity may not only result from the production of acidic metabolites, but also from other proteinaceous-excreted metabolites, such as bacteriocin.

In conclusion, this study has shown for the first time that IAP against GBS has a significant influence on the early bifidobacterial pattern of newborns, both quantitatively and qualitatively. Further studies are necessary to evaluate the long-term effects of IAP on the newborn gut microbiota. In addition, only a complete analysis of the whole microbiota in newborns born by IAP-treated mother via high-throughput sequencing will allow a full understanding of the impact of the prophylaxis. Moreover, this work has preliminary explored the possibility of using a non chemotherapeutic approach for the prevention of GBS infections, i.e., the use of *Bifidobacterium* spp. strains which can also be beneficial for the newborn.

References

Aloisio I, Santini C, Biavati B, Dinelli G, Cencič A, Chingwaru W, Mogna L, Di Gioia D (2012) Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns. Appl Microbiol Biotechnol 96:1561–1576

Al-Taiar A, Hammoud M, Thalib L, Isaacs D (2011) Pattern and etiology of culture-proven early onset neonatal sepsis: a five year prospective study. Int J Infec Dis 15:631–634

Altschul S, Gish W, Miller W, Myers E, Lipman D (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Amaretti A, di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A (2013) Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. Appl Microbiol Biotechnol 97:809–817

Ammor MS, Flórez AB, van Hoek AHAM, de Los Reyes-Gavilán CG, Aarts HJM, Margolles A, Mayo B (2008) Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. J Mol Microbiol Biotechnol 14:6–15

Bäckhed F, Fraser CM, Ringel Y, Sanders ME, Sartor RB, Sherman PM, Versalovic J, Young V, Finlay BB (2012) Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. Cell Host Microbe 12:611–622

Benzecri J (1992) Correspondance analyses handbook. CRC Press, Boca Raton, Florida

Bizzarro M, Dembry L, Baltimore R, Gallagher P (2008) Changing patterns in neonatal *Escherichia coli* sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. Pediatrics 121:689–696

Bush K, Courvalin P, Dantas G, Davies J, Eisenstein B, Huovinen P, Jacoby GA, Kishony R, Kreiswirth BN, Kutter E, Lerner SA, Levy S, Lewis K, Lomovskaya O, Miller JH, Mobashery S, Piddock LJV, Projan S, Thomas CM, Tomasz A, Tulkens PM, Walsh TR, Watson JD, Witkowski J, Witte W, Wright G, Yeh P, Zgurskaya HI (2011) Tackling antibiotic resistance. Nat Rev Microbiol 9:894–896

Cani PD, Delzenne NM (2011) The gut microbiome as therapeutic target. Pharmacol Ther 130:202–212

Castillo M, Martín-Orúe SM, Manzanilla EG, Badiola I, Martín M, Gasa J (2006) Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet Microbiol 114:165–170

Chiang S-S, Pan T-M (2012) Beneficial effects of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 and its fermented products. Appl Microbiol Biotechnol 93:903–916

D'Aimmo MR, Modesto M, Biavati B (2007) Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. Int J Food Microbiol 115:35–42

Di Gioia D, Aloisio I, Mazzola G, Biavati B (2014) Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. Appl Microbiol Biotechnol 98:563–577

Dotterud CK, Storrø O, Johnsen R, Oien T (2010) Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. Br J Dermatol 163:616–623

Ferrieri P, Wallen L (2012) Neonatal bacterial sepsis. In: Gleason CA, Devaskar S (eds) Avery's disease of the newborn, 9th edn. Elsevier Saunders, Philadelphia, p 538–550

Fuligni F, Gueimende M, Margolles A, De Bellis G, van O'Toole PW, Sinderen D, Marchesi JR, Ventura M (2012) Diversity of bifidobacteria within the infant gut microbiota. PLoS ONE 7:e36957

Grice EA, Segre JA (2012) The human microbiome: our second genome. Annu Rev Genomics Hum Genet 13:151–170

Keski-Nisula L, Kyynäräinen H-R, Kärkkäinen U, Karhukorpi J, Heinonen S, Pekkanen J (2013) Maternal intrapartum antibiotics and decreased vertical transmission of *Lactobacillus* to neonates during birth. Acta Paediatr 102:480–485

Klappenbach J, Saxman P, Cole J, Schmidt T (2001) rrnDB: the ribosomal RNA operon copy number database. Nucleic Acids Res 29:181–184

Lee Z, Bussema C, Schmidt T (2009) rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res 37:489–493

Malinen E, Kassinen A, Rinttila T, Palva A (2003) Comparison of real-time PCR with SYBR Green I or 5′-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiology 149:269–277

Mangin I, Suau A, Gotteland M, Brunser O, Pochart P (2010) Amoxicillin treatment modifies the composition of *Bifidobacterium* species in infant intestinal microbiota. Anaerobe 16:433–438

Mättö J, van Hoek AHAM, Domig KJ, Saarela M, Floréz AB, Brockmann E, Amtmann E, Mayo B, Aarts HJM, Danielsen M (2007) Susceptibility of human and probiotic *Bifidobacterium* spp. to selected antibiotics as determined by the Etest method. Int Dairy J 17:1123–1131

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Anton Leeuw 73:127–141

O'Sullivan A, He X, McNiven EMS, Haggarty NW, Lönnerdal B, Slupsky CM (2013) Early diet impacts infant rhesus gut microbiome, immunity, and metabolism. J Proteome Res 12:2833–2845

Palmer C, Bik E, DiGiulio D, Relman D, Brown P (2007) Development of the human infant intestinal microbiota. PLoS Biol 5:1556–1573

Penders J, Thijs C, Vink C, Stelma F, Snijders B, Kummeling I, van den Brandt P (2006) Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118:511–521

Phillips EA, Tapsall JW, Smith DD (1980) Rapid tube CAMP test for identification of *Streptococcus agalactiae* (Lancefield group B). J Clin Microbiol 12:135–137

Puopolo K, Madoff L, Eichenwald E (2005) Early-onset group B streptococcal disease in the era of maternal screening. Pediatrics 115:1240–1246

Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97:1166–1177

Sanders M, Akkermans L, Haller D, Hammerman C, Heimbach J, Hörmannsperger G, Huys G (2010) Safety assessment of probiotics for human use. Gut Microbes 1:164–185

Santini C, Baffoni L, Gaggia F, Granata M, Gasbarri R, Di Gioia D, Biavati B (2010) Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. Int J Food Microbiol 141:S98–S108

Sanz Y, Sánchez E, Marzotto M, Calabuig M, Torriani S, Dellaglio F (2007) Differences in faecal bacterial communities in celiac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. FEMS Immunol Med Microbiol 51:562–568

Satokari RM, Vaughan EE, Akkermans ADL, Saarela M (2001) Bifidobacterial diversity in human feces detected by genus- specific pcr and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67:504–513

Satokari R, Vaughan E, Favier F, Dore J, Edwards C, de Vos W (2002) Diversity of *Bifidobacterium* and *Lactobacillus* spp. in breast-fed and formula-fed infants as assessed by 16S rDNA sequence differences. Microb Ecol Health Dis 14:97–105

Savino F, Cordisco L, Tarasco V, Locatelli E, Di Gioia D, Oggero R (2011) Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC Microb 11:157

Schloissnig S, Arumugam M, Sunagawa S, Mitreva M, Tap J, Zhu A, Waller A, Mende DR, Kultima JR, Martin J, Kota K, Sunyaev SR, Weinstock GM, Bork P (2013) Genomic variation landscape of the human gut microbiome. Nature 493:45–50

Sharon I, Morowitz MJ, Thomas BC, Costello EK, Relman DA, Banfield JF (2013) Time series community genomics analysis reveals rapid shifts in bacterial species, strains, and phage during infant gut colonization. Genome Res 23:111–120

Solís G, de Los R-GC, Fernández N, Margolles A, Gueimonde M (2010) Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. Anaerobe 16:307–310

Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M (2012) Diversity of bifidobacteria within the infant gut microbiota. PLoS One 7:e36957

Wickens K, Black PN, Stanley TV, Mitchell E, Fitzharris P, Tannock GW, Purdie G, Crane J (2008) A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol 122:788–794

Zárate G, Nader-Macias ME (2006) Influence of probiotic vaginal lactobacilli on in vitro adhesion of urogenital pathogens to vaginal epithelial cells. Lett Appl Microbiol 43:174–180

PAPER 6

Influence of intrapartum antibiotic prophylaxis for Group B *Streptococcus* and type of feeding on gut microbiota during the first month of life

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Abstract

The effect of intrapartum antibiotic prophylaxis (IAP) for Group B *Streptococcus* (GBS) on bacterial colonization of the infant's gut has not been investigated extensively.

We aimed to evaluate the effect of IAP on gut microbiota in healthy term infants, also exploring the influence of type of feeding.

Healthy term infants, whose mothers had been screened for GBS in late gestation, were divided into two groups: infants born to GBS-positive mothers who had received IAP vs. controls. Neonatal fecal samples were collected at 7 and 30 days of life; DNA was extracted and quantification of selected microbial groups (*Lactobacillus* spp., *Bifidobacterium* spp. and *Bacteroides fragilis*) was performed by real-time PCR.

Bifidobacteria count was significantly lower in the IAP group at 7 days of life (independent-samples Mann-Whitney U test; median [interquartile range] 6.01 Log CFU/g [5.51-6.98] vs. 7.80[6.61-8.26]; p=0.000). No differences in *Bifidobacteria* count at 30 days or in *Lactobacilli* and *Bacteroides fragilis* counts at any time point were documented.

Hierarchical regression analysis showed that, at 7 days of life, infants who had not received IAP and were exclusively HM-fed had higher counts of *Bifidobacteria*. Furthermore, regardless of IAP treatment, infants fed exclusive HM had higher *Lactobacillus spp.* counts both at 7 and 30 days of life.

IAP alters gut microflora by reducing the count of *Bifidobacteria*, which is further affected in infants receiving formula feeding. Whether these alterations could have long-term consequences on health and disease requires further investigation.

Key words Microbiota; infant; intrapartum antibiotic prophylaxis, Group B *Streptococcus*; human milk.

Introduction

The colonization of the gastrointestinal (GI) tract is thought to begin during the birth process, when the infant's gut is exposed to maternal and environmental bacteria (Thompson-Chagoyán et al. 2007). However, recent studies performed in preterm foetuses and infants have shown that amniotic fluid and meconium are not sterile, thus suggesting an intrauterine origin of gut microbiota (DiGiulio et al. 2008; Mshvildadze et al. 2010). At birth, the neonatal GI tract is rapidly colonized by bacteria from the mother and the environment; the first colonizers are generally aerobes and facultative anaerobes (Jauréguy et al. 2004; Di Gioia et al. 2014), followed by strict anaerobes such as *Bifidobacterium* spp., *Bacteroides* spp., and *Clostridium* spp. (Aloisio et al. 2014). The composition of gut microbiota is influenced by several factors, including mode of delivery, gestational age (GA), maternal microbiota of the intestine, vagina and epidermis, hospitalization after birth, type of infant feeding and use of antibiotics and probiotics (Penders et al. 2006; Savino et al. 2011; Jost et al. 2012; Aloisio et al. 2014). Gut microbiota of term infants, born by vaginal delivery (VD) and exclusively breastfed, is considered to be ideally healthy, with its low count of *C. difficile* and *E. coli* and high number of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* [10].

Group B *Streptococcus* (GBS), mainly represented by *Streptococcus agalactiae* strains, is one of the most important causes of infection and sepsis in the neonatal period. Infants born by VD may acquire GBS during the birth process from maternal vagina, cervix or rectum, where it resides in approximately 10-20% of pregnant women [11]. The incidence of early-onset GBS sepsis declined significantly in the last decade, due to the introduction of GBS universal screening during late pregnancy [12] and consequent intrapartum antibiotic prophylaxis (IAP) in GBS-positive women [13].

Recent data suggest that the use of antibiotics in early life can impair the balance between health and disease later in life by altering commensal gut microbiota [14]. The effect of IAP on bacterial colonization of the infant's gut has not been investigated extensively [4,15]. Studies performed up to now, mainly based on the use of culture-based techniques, which are known to have several limitations in particular in counting and isolation of anaerobic bacteria [16], showed that IAP does not increase the amount of antibiotic-resistant enterobacteria [4], but could reduce vertical transmission of lactic acid bacteria from IAP-treated mothers to the neonates [15]. Both studies remarked the requirements of further investigations to clarify the effect of IAP on the newborn microbiota.

In a preliminary study, we evaluated by means of molecular techniques the effect of IAP in a relatively small sample of exclusively breast-fed term infants born by VD, showing, at 7 days of

life, a significant decrease of the bifidobacteria counts in newborns born to IAP-treated mother. Moreover, IAP determined a decrement in the frequency of some bifidobacterial species with respect to newborns born to non-treated mothers [6].

The aim of the present paper was thus to evaluate these differences in further details, expanding the initial number of subjects and following up infants until one month of age. The influence of type of feeding on microbiota composition was also explored.

Materials and Methods

The study was performed in the Nursery of S. Orsola-Malpighi Hospital in Bologna, Italy, and was approved by the Institutional Ethic Committee (study ID 12/2013/U/Oss).

Patients

Between October 2012 and June 2013, healthy term infants, born by VD, with birth weight adequate for GA (AGA), and whose mothers had been screened for GBS at 35-37 weeks gestation, were enrolled in the study. The exclusion of preterm or small/large for GA infants, infants born by caesarean section, and infants admitted to the Neonatal Intensive Care Unit was made in order to minimize potential confounding factors [17].

Infants were excluded also in the following cases:

- the mother had received any antibiotic other than IAP in the 4 weeks before delivery;
- maternal IAP was performed for reasons other than GBS positivity (i.e. prolonged rupture of membranes in GBS-negative women);
- maternal IAP was performed with antibiotics other than ampicillin, such as erythromycin;
- the infant had major congenital malformations;
- the infant developed signs of infection and/or received any antibiotic treatment after birth;
- the infant had, or developed at birth, any serious clinical conditions that contraindicated the participation in the study.

Infants were divided into two groups according to maternal GBS status and IAP:

- IAP group: infants born to GBS-positive mothers who had received IAP. According to the Institutional treatment protocol for GBS prophylaxis (derived from CDC guidelines [12]), iv ampicillin was given every 4 hours until delivery (first dose 2 g, following doses 1 g each).
- Control group: infants born to GBS-negative mothers, who thus did not receive any antibiotic treatment before/at delivery.

Written informed consent was obtained from each infant's parent/legal guardian when the infant was about to be discharged from the nursery (48-72 hours of life). Patients' characteristics, including GA, birth weight, gender, and Apgar score at 1' and 5' after birth, were summarized in a specific case report form.

Faecal samples' collection and analysis

Follow-up visits were performed at 7 and 30 days of life. At each visit, information on infants' weight gain, clinical conditions, and on-going treatments (i.e. use of prebiotics, probiotics, antibiotics) was collected. Furthermore, the characteristics of the infants' feeding (exclusive breastfeeding, exclusive formula feeding or mixed feeding) were recorded.

Fecal samples were collected at each follow-up visit. After collection, they were put into numbered screw-capped sterile plastic containers, which were immediately frozen at -80 °C, until they were processed for DNA extraction.

Microbiological analyses were performed at the Laboratory of Microbiology, Department of Agricultural Sciences, University of Bologna, according to previously published methods [6]. Investigators who performed the analyses were blind to group identity of the infants.

Two hundred milligrams of faeces were used for DNA extraction using the QIAamp DNA Stool Mini Kit (QIAGEN, West Sussex, UK). Extracted DNA was stored at -80 °C. The purity and concentration of extracted DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland).

Quantification of selected microbial groups (*Bifidobacterium* spp., *Lactobacillus* spp., and *Bacteroides fragilis* group) was carried out with real-time PCR. The assays were performed as previously described [6]. Data obtained from amplification were transformed to obtain the number of bacterial cells per gram of faeces, expressed as Log colony forming unit (CFU)/g.

Statistical analysis

Data were analysed using IBM SPSS Statistic version 20.0.0 (IBM Corporation, IBM Corporation Armonk, New York, United States).

Baseline characteristics in the IAP and control groups were compared using the independent-samples Mann-Whitney U test for continuous variables and chi-square test for categorical variables.

The influence of IAP on faecal bacterial count at 7 and 30 days of life was evaluated using the independent-samples Mann-Whitney U test.

Furthermore, multiple regression analysis was performed in order to estimate the effect of IAP on faecal bacterial count after controlling for type of infant feeding. Specifically, a hierarchical regression analysis was performed: IAP was entered first, followed by type of feeding. For the analysis, feeding type was coded as a binary categorical variable: exclusive breastfeeding vs. any formula feeding (this latter includes infants receiving exclusive formula or a variable proportion of breast milk and formula). A p value <0.05 was considered as statistically significant.

Results

Patients

During the study period, 84 newborns were recruited (35 in the IAP and 49 in the control group). Neonatal characteristics did not differ between infants in the IAP and control groups (Table 1).

Table 1. Baseline characteristics of the enrolled infants

	IAP group	**Control group**	**p value**
# of infants	35	49	
Gestational age, weeks	40 (39-40.5)	40 (39-41)	.308
Birth weight, grams	3370 (3130-3680)	3335 (3049-3583)	.571
Gender (male/female)	17/18	29/20	.229
Apgar score at 1'	9 (9-9.25)	9 (9-10)	.393
Apgar score at 5'	10 (10-10)	10 (10-10)	.340

Values are expressed as median (interquartile range) were appropriate.
IAP: intrapartum antibiotic prophylaxis.

All the recruited infants were evaluated at 7 and 30 days after birth. The characteristics of the infants at the two follow-up visits are shown in Table 2. No difference between groups in terms of weight gain and rate of exclusive breastfeeding was documented. None of the infants was receiving, or had received since birth, any treatment with prebiotics, probiotics, and antibiotics.

Table 2. Characteristics of the enrolled infants evaluated at 7 and 30 days of life.

	IAP group (N=35)	Control group (N=49)	p value
7-days evaluation			
Weight gain from birth, g/day	-82 (-160 - +30)	-57 (-165 - +10)	.982
Feeding • Exclusive breastfeeding • Any formula feeding	 26 (74.3%) 9 (25.7%)	 30 (61.2%) 19 (38.8%)	.247
30-days evaluation			
Weight gain from birth, g/day	850 (661 - 1005)	680 (543 - 943)	.335
Feeding • Exclusive breastfeeding • Any formula feeding	 22 (62.9%) 13 (37.1%)	 29 (59.2%) 20 (40.8%)	.822

Values are expressed as median (interquartile range), or number (percentage), as appropriate.
IAP: intrapartum antibiotic prophylaxis.

Influence of IAP on faecal bacterial counts

The count of *Bifidobacterium* spp. was significantly lower in the IAP group than in the control group at 7 days of life (independent-samples Mann-Whitney U test; median [interquartile range] 6.01 Log CFU/g [5.51-6.98] vs. 7.80[6.61-8.26], respectively; p=0.000), while no difference was documented at 30 days (8.41 [7.71 – 8.80] vs. 8.39 [7.96 - 8.86], respectively; p=0.842). No difference was documented between the two groups at any time point in the count of *Lactobacillus* spp. (5.56 [4.94 - 6.14] vs. 5.45 [4.81 - 6.14] at 7 days; p=0.518. 5.29 [4.68 - 6.01] vs. 5.25 [4.60 - 6.15] at 30 days; p=0.818) and *Bacteroides fragilis* group (7.71 [5.80 - 9.33] vs. 7.75 [5.87 - 9.61] at 7 days; p=0.618. 7.36 [5.80 - 9.09] vs. 8.51 [5.86 - 9.37] at 30 days; p=0.479).

Hierarchical multiple regression was performed for each bacterial group, both at day 7 and day 30. The results of these analyses for *Bifidobacterium spp.* and *Lactobacillus spp.* are provided in Table 3.

Table 3. Results of hierarchical multiple regression analysis.

***Bifidobacterium* spp.**							
			B	SE B	β	t	P
7 DOL	Step 1	(constant)	7.593	.176		43.077	.000
		IAP[a]	-1.298	.273	-.465	-4.754	.000
	Step 2	(constant)	7.926	.198		39.962	.000
		IAP[a]	-1.410	.262	-.505	-5.384	.000
		Feeding[b]	-.858	.274	-.294	-3.133	.002
30 DOL	Step 1	(constant)	8.294	.176		46.994	.000
		IAP[a]	-.245	.273	-.098	-.896	.373
	Step 2	(constant)	8.363	.210		39.780	.000
		IAP[a]	-.251	.275	-.101	-.915	.363
		Feeding[b]	-.169	.277	-.067	-.610	.544
***Lactobacillus* spp.**							
			B	SE B	β	t	P
7 DOL	Step 1	(constant)	5.516	.146		37.704	.000
		IAP[a]	.113	.224	.056	.505	.615
	Step 2	(constant)	5.729	.170		33.645	.000
		IAP[a]	.036	.221	.018	.161	.872
		Feeding[b]	-.527	.230	-.252	-2.290	.025
30 DOL	Step 1	(constant)	5.351	.169		31.741	.000
		IAP[a]	.020	.258	.008	.076	.940
	Step 2	(constant)	5.672	.192		29.513	.000
		IAP[a]	-.021	.246	-.009	-.086	.932
		Feeding[b]	-.754	.248	-.324	-3.036	.003

DOL: days of life; IAP: intrapartum antibiotic prophylaxis

[a] IAP was coded as 0=no IAP, 1=IAP

[b] Feeding was coded as 0=exclusively HM, 1=any formula feeding

At 7 days of life, IAP and feeding type were significantly associated with *Bifidobacterium spp.* count, with higher counts in infants who had not received IAP and were exclusively HM-fed. IAP accounted for approximately 22% of the variance of the outcome (R^2=.216 in step 1) and feeding type contributed for an additional 8% (R^2=.301 in step 2). At 30 days of life, *Bifidobacterium spp.* count was unrelated to IAP or feeding type.

Hierarchical regression analysis confirmed that IAP was unrelated to *Lactobacillus spp.* counts either at 7 and 30 days of life. However, this analysis showed a significant effect of feeding type on *Lactobacillus spp.* counts: regardless of IAP treatment, infants fed exclusive HM had higher *Lactobacillus spp.* counts both at 7 and 30 days of life. Feeding type gave the main contribution to the variability of the outcome (approximately 6% at 7 days [R^2=.003 in step 1 and R^2=.065 in step 2] and 11% at 30 days [R^2=.000 in step 1 and R^2=.105 in step 2]).

No significant influence of IAP or feeding type was documented for *Bacteroides fragilis* group, either at 7 or 30 days of life (data not shown).

Discussion

Three groups of bacteria were monitored in this work: members of the *Bifidobacterium* genus, which were shown to decrease at 7 days of life in infants born to mothers who received IAP with respect to those born to untreated mothers [6], members of the *Lactobacillus* genus, which showed a reduced vertical transmission from IAP treated mothers to newborns [15], and members of the *B. fragilis* group. This group comprises species like *Bacteroides thetaiotaomicron* and *B. fragilis*, which have recently been shown to be pioneer bacteria in the majority of neonates, particularly the breast-fed ones [7]. The results of the present study show that prenatal antibiotic treatment in GBS-positive mothers has an early and transient influence on the infant's gut microbiota: specifically, faecal count of *Bifidobacteria* is reduced by maternal IAP in the first week of life, as already shown in a preliminary study [6], but gets back to normal at one month of life. Furthermore, in infants born to GBS-positive mothers, an additional negative factor in terms of *Bifidobacteria* colonization is given by the use of formula feeding. The counts of *Lactobacilli* and *Bacteroides fragilis* group are not influenced by IAP: however, regardless IAP, exclusively HM-fed infants have a higher lactobacilli count both at 7 and 30 days of life.

The introduction of universal screening for GBS and consequent IAP which followed the CDC updated guidelines [12] has dramatically reduced the incidence of early-onset GBS sepsis both in the US and in Europe, where most countries launched national guidelines for GBS prevention [11]. Despite the clinical benefit of IAP, however, little is known on how it affects neonatal bacterial gut colonization and whether alterations of gut microbiota related to IAP could have short and long-term consequences in terms of health and disease.

Previous studies on this topic have important limitations, such as the reduced number of samples considered, the non-standardization of potential confounding factors (such as mode of delivery, GA, and prolonged rupture of membranes) and the use of culture dependent techniques which

may have drawbacks when counting faecal bacteria, in particular anaerobic ones [16]. Our study was therefore designed to overcome these problems: a highly-selected population of healthy, AGA, and term infants born by VD, was recruited and microbial populations were counted with the use of molecular techniques. To our knowledge, this is the first study investigating the effect of IAP on gut microbiota in the first month of life by means of molecular techniques. Only one previous study was focused specifically on IAP [4]: twenty-five 3-days-old infants born to GBS-positive mothers who had received iv IAP with amoxicillin were compared to 25 controls, matched for GA, mode of delivery, and type of feeding. No differences in the count of *Bifidobacteria* and *Bacteroides* were documented; however, faecal samples were analysed by culture-dependent methods and it was not possible to document any specific effect of feeding. In the study by Keski-Nisula [15], which investigated vertical transmission of *Lactobacillus* spp., IAP and longer rupture of membranes were associated with a lower transmission rate of *Lactobacilli*. However, the study was not comparable to ours, because a relatively unselected population of term infants was recruited and the *Lactobacillus* population was studied using a neonatal oral swab and analysed by culture-based methods.

One further study [18] examined by means of molecular techniques the influence of prenatal and neonatal antibiotic treatments on infants' gut microbiota over the first two months of life: similarly to the results of our study, colonization by *Bifidobacteria* was initially attenuated in infants exposed to prenatal or neonatal antibiotics and got back to normal at 2 months of life. However, the group receiving prenatal antibiotic treatment was formed of only three caesarean-delivered subjects. Any alteration on the development of gut microbiota in early life is presumably associated with a divergent immunological starting point in the host, with potential implications for the development of disease later in life [14]. Several events in early life can lead to a perturbation in the physiological development of a healthy microbiota. In the present study, type of feeding was found to have a great impact on gut colonization in the first days of life: exclusive HM feeding had a positive and persistent effect on the count of *Lactobacilli*, which was independent from antibiotic exposure; furthermore, the use of formula had a negative effect, which was additional to the effect of IAP, on the count of *Bifidobacteria* at 7 days of life.

Although available data regarding differences in gut microbiota composition in breastfed and formula-fed infants are often contradictory [19], recent results obtained with culture-independent methods showed that infants fed HM have higher count of *Bifidobacteria* compared to formula-fed infants [20, 21].

HM is a complex biofluid which has both probiotic and prebiotic properties: it represents a unique source of bacteria, such as *Lactobacilli*, which are able to colonize the infant's gut and to

promote health benefits for the host [22], and also contains specific oligosaccharides which exert a prebiotic effect on gut microbiota, stimulating beneficial microorganisms such as *Bifidobacteria* and *Lactobacilli* [23]. The development of gut microbiota is driven by the so-called "pioneer bacteria": in this perspective, alterations in the composition of gut microbiota in early life potentially have strong implications in terms of later health and disease. The results of our study show that IAP alters the infant's microbiota by reducing the count of *Bifidobacteria*, and that this is further affected in infants receiving formula feeding. Whether these alterations could have long-term consequences on health and disease is unknown. However, the promotion of exclusive breastfeeding appears to be important for reducing the alterations in the count of *Bifidobacteria* induced by IAP, and also for promoting the infant's colonisation with beneficial bacteria such as *Lactobacilli*.

These findings also suggest that further studies should investigate the opportunity of giving a formulation containing potential probiotic bacteria such as *Lactobacilli* and *Bifidobacteria* in the first weeks of life to infants born to GBS-positive mothers and not receiving exclusive breastfeeding.

References

1. Thompson-Chagoyán OC, Maldonado J, Gil A. Colonization and impact of disease and other factors on intestinal microbiota. Dig Dis Sci. 2007;52(9):2069–77.

2. DiGiulio DB, Romero R, Amogan HP, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PLoS One. 2008;3(8):e3056.

3. Mshvildadze M, Neu J, Shuster J, Theriaque D, Li N, Mai V. Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. J Pediatr. 2010;156(1):20–5.

4. Jauréguy F, Carton M, Panel P, Foucaud P, Butel M-J, Doucet-populaire F. Effects of Intrapartum Penicillin Prophylaxis on Intestinal Bacterial Colonization in Infants. J Clin Microbiol. 2004;42(11):5184–5188.

5. Di Gioia D, Aloisio I, Mazzola G, Biavati B. Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. Appl Microbiol Biotechnol. 2014;98(2):563–77.

6. Aloisio I, Mazzola G, Corvaglia LT, et al. Influence of intrapartum antibiotic prophylaxis against group B Streptococcus on the early newborn gut composition and evaluation of the anti-Streptococcus activity of Bifidobacterium strains. Appl Microbiol Biotechnol. 2014;98(13):6051–60.

7. Jost T, Lacroix C, Braegger CP, Chassard C. New insights in gut microbiota establishment in healthy breast fed neonates. PLoS One. 2012;7(8):e44595.

8. Penders J, Thijs C, Vink C, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 2006;118(2):511–21.

9. Savino F, Roana J, Mandras N, Tarasco V, Locatelli E, Tullio V. Faecal microbiota in breast-fed infants after antibiotic therapy. Acta Paediatr. 2011;100(1):75–8.

10. Johnson CL, Versalovic J. The human microbiome and its potential importance to pediatrics. Pediatrics. 2012;129(5):950–60.

11. Rodriguez-Granger J, Alvargonzalez JC, Berardi a, et al. Prevention of group B streptococcal neonatal disease revisited. The DEVANI European project. Eur J Clin Microbiol Infect Dis. 2012;31(9):2097–104.

12. Verani J, McGee L, Schrag S, CDC. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC, 2010. MMWR Recomm Rep. 2010;59:1–32.

13. Jordan HT, Farley MM, Craig A, et al. Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease: a multistate, population-based analysis. Pediatr Infect Dis J. 2008;27(12):1057–64.

14. Zeissig S, Blumberg RS. Life at the beginning: perturbation of the microbiota by antibiotics in early life and its role in health and disease. Nat Immunol. 2014;15(4):307–10.

15. Keski-Nisula L, Kyynäräinen H-R, Kärkkäinen U, Karhukorpi J, Heinonen S, Pekkanen J. Maternal intrapartum antibiotics and decreased vertical transmission of Lactobacillus to neonates during birth. Acta Paediatr. 2013;102(5):480–5.

16. O'Toole PW, Claesson MJ (2010) Gut microbiota: Changes throughout the lifespan from infancy to elderly. Int Dairy J 20:281–291.

17. Eggesbø M, Moen B, Peddada S, et al. Development of gut microbiota in infants not exposed to medical interventions. APMIS. 2011;119(1):17–35.

18. Tanaka S, Kobayashi T, Songjinda P, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. FEMS Immunol Med Microbiol. 2009;56(1):80–7.

19. Adlerberth I, Wold a E. Establishment of the gut microbiota in Western infants. Acta Paediatr. 2009;98(2):229–38.

20. Bezirtzoglou E, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). Anaerobe. 2011;17(6):478–82.

21. Le Huërou-Luron I, Blat S, Boudry G. Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. Nutr Res Rev. 2010;23(1):23–36.

22. Albesharat R, Ehrmann M a, Korakli M, Yazaji S, Vogel RF. Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. Syst Appl Microbiol. 2011;34(2):148–55.

23. Musilova S, Rada V, Vlkova E, Bunesova V. Beneficial effects of human milk oligosaccharides on gut microbiota. Benef Microbes. 2014;5(3):273–83.

PAPER 7

High-throughput sequencing and q-PCR approach to study early gut microbiota perturbations following intrapartum antibiotic prophylaxis to prevent group B streptococcal disease

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Abstract

Introduction: The aim of this study was to use high-throughput pyrosequencing in combination with quantitative PCR (q-PCR) to thoroughly examine the effects of intrapartum antibiotic prophylaxis (IAP) against group B Streptococcus (GBS) on the infant gut microbiota.

Materials and methods: Bacterial DNA was extracted from twenty-six infant's feces at day 7 and 30. Q-PCR of total bacteria, *Lactobacillus* spp., *Bidobacterium* spp. and *Bacteroides fragilis* group was performed as well as sequencing of the V3-V4 hypervariable region of the 16S rRNA gene. Infants were divided into 4 groups: Breast-fed (BF) born to GBS negative mothers (1) and to GBS positive, IAP treated mothers (2); infants fed with a mixture of breast milk and formula milk (mixed-fed, MF) born to GBS negative mothers (3) and GBS positive, IAP treated mothers (4).

Results: Q-PCR revealed that *Bifidobacterium* spp. was significantly reduced in infants born to IAP treated mothers, both BF and MF, at day 7 of life, while other bacterial groups were unaffected. High-throughput sequencing showed a significant reduction of microbial richness and biodiversity at day 7 in the IAP treated groups (irrespective of feeding type), whereas differences were recovered at day 30. The proportions of *Bifidobacteriaceae* ($P<0.001$) and *Enterobacteriaceae* ($P<0.044$) were significantly lower and higher, respectively, in breast-fed IAP treated infants compared to control group at day 7.

Conclusions: This study has definitively demonstrated the negative and short term consequence of IAP on newborn gut bacterial population, which are recovered after 1 month irrespective of the feeding type.

Key Words: high-throughput pyrosequencing, q-PCR, *Streptococcus*, intrapartum antibiotic prophylaxis.

Introduction

Streptococcus agalactiae, also known as Group B Streptococcus (GBS), is a gram-positive commensal bacterium which resides in the gastrointestinal and genitourinary tract of many of the population asymptomatically. However, in pregnant women, GBS can be vertically transmitted to the neonate where an estimated 1-2% develop early-onset GBS disease.[1,2] In Europe, the prevalence of GBS colonisation among pregnant women varies between 6.5% and 36%, with one third of studies reporting rates of 20% or greater.[3] GBS infections among infants have been implicated as a leading cause of respiratory disease, sepsis, meningitis and bacteraemia.[4-6] Risk factors for neonatal infection include prematurity, prolonged rupture of membranes (> 18 hours) and an intrapartum temperature (> 38°C).[7]

To minimize the risk of early-onset neonatal disease due to GBS, the Centres for Disease Control and Prevention (CDC) have recommended the practise of universal GBS screening of pregnant women at 35-37 weeks of gestation and intrapartum antibiotic prophylaxis (IAP) for women positive for GBS.[8] The introduction of these guidelines has seen a reduction in the vertical transmission of GBS and consequently early-onset GBS disease, with a reduction in mortality from 50% to 4%. [9,10]

While the benefits of the use of IAP in the prevention of early-onset GBS transmission have been acknowledged,[11,12] the impact of IAP use on the development of the infant gut microbiota has been scarcely studied. Infants firstly encounter maternal vaginal and faecal microbiota during birth and secondly acquire microorganisms from the external environment such as mammary gland, mouth and skin.[13,14] Briefly, the first colonizing bacteria are facultative anaerobic bacteria, mainly staphylococci, streptococci, enteroccoci, and enterobacteria,[15] after establishment of a reducing environment, anaerobic bacteria such as *Bacteroides*, *Bifidobacterium* and *Clostridium* spp. dominate. Biodiversity and microbial richness continue to increase until the conclusion of weaning when the microbiota becomes similar to that of the adult.[16-19] During this short temporal window, a number of factors can perturb colonization; these include mode of delivery, neonatal intensive care environment, sanitary conditions, feeding choice, preterm vs. full term, maternal weight, diet and antibiotic use.[20-24]

Studies on the possible effects of maternal IAP against GBS on the microbiota composition in infants have reported a reduced vertical transmission of lactic acid bacteria from IAP-treated mothers [25] and an early reduction in *Bifidobacterium* spp..[26] However, the methodologies applied (plate counts, q-PCR on selected microbial groups and PCR-DGGE) did not allow an exhaustive analysis of the whole microbiota composition. Therefore, the aim of our study is to use high-

throughput pyrosequencing (Illumina MiSeq System) in combination with q-PCR to thoroughly examine the impact of maternal IAP on the entire microbiota composition in the first month of life. In addition, the effect of feeding regime, i.e. exclusive breast-feeding versus mixed feeding was also investigated in these infants.

Materials and Methods

Study design and samples collection

Ethical approval for the study design and protocol were received from the Comitato Etico Indipendente dell'Azienda Ospedaliero-Universitaria di Bologna, Policlinico S. Orsola-Malpighi (document number 12/2013/U/Oss approved on March 12, 2013).

Twenty six infants born at the Neonatal Intensive Care Unit of the S. Orsola-Malpighi Hospital of Bologna were recruited between April 2013 and December 2013. Informed written consent for participation in the study was obtained from all parents. Infants were born at term by vaginal delivery, and at birth weighed between 2.5 - 4.0 kg and did not receive any perinatal antibiotic or probiotic/prebiotic treatment. Each infant belonged to one of four groups, (Table 1). Briefly groups were comprised of: 1) BF-C: Breast-fed (BF) infants born to GBS negative mothers (control); 2) BF-IAP: BF infants born to GBS positive, IAP treated mothers; 3) MF-C: Mixed-fed (MF) infants born to GBS negative mothers (control); 4) MF-IAP: infants born to GBS positive, IAP treated mothers.

Table 1: Characteristics of infants in this study

Newborns[#]	Feeding method[a]	IAP[b]	Group
1): 7 (5 M[1] and 2 F[2])*	Breast-fed	NO	BF-C
2): 7 (5 M and 2 F)	Breast-fed	YES	BF-IAP
3): 6 (1 M and 5 F)	Mixed-fed	NO	MF-C
4): 6 (2 M and 4 F)	Mixed-fed	YES	MF-IAP

[a]Breast-fed = exclusive breast-feeding; Mixed-fed = combination of formula and breast milk with at least 50% of formula feeding (it did not contain probiotics or prebiotics).
[b]NO = control sample; YES = IAP treated.
*one sample not available at 7 days of life.
[1]M= males; [2]F= females. [#] Vaginal delivery.

The GBS positive women were defined at the gestational age between 35 to 37 weeks when a vaginal and rectal swab was performed. Swabs were analysed through the strep B Carrot Broth™, a one-step method for cultivation and identification of haemolytic strains of Group B Streptococcus (Hardy Diagnostics, Santa Maria, CA).

Women who tested positive for GBS were treated with 2g of ampicillin (Amplital®) at the beginning of labour followed by 1g every 4 hours until delivery.[12] All IAP treated women considered in this study received a maximum of 4g of ampicillin.

Infant faecal samples were collected at day 7 and day 30 of life and were immediately frozen at -80 °C until analysis.

DNA extraction from infant samples

Total bacterial DNA was extracted from 200 mg of stool using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions with a slight modification: an additional incubation at 95 °C for 10 min of the stool sample with lysis buffer to improve the efficiency of bacterial cell rupture of Gram-positive bacteria. Extracted DNA was stored at -80 °C.

Quantitative PCR

Absolute quantification of *Lactobacillus* spp., *Bidobacterium* spp., *Bacteroides fragilis* group and total bacterial numbers in infant fecal samples was determined by q-PCR. Standard curves were constructed using the PCR products of the 16S rRNA gene of *Lactobacillus brevis* DSM 20054^T, *Bifidobacterium breve* DSM 20213^T, *Bifidobacterium longum* subsp. *longum* DSM 20219^T and *Bacteroides fragilis* DSM 2151^T. The PCR products were purified with NucleoSpin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and then quantified spectrophotometrically (Infinite® 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland). Standard curves for each microbial group were established using 10^2 to 10^6 copies 16S rRNA/µl. Details of primer sets utilised are shown in Table S1.

Table S1: Sequences of the primers used and q-PCR equations

Bacterial group	Primer (5'-3')	Reference	Equation	R^2
Lactobacillus spp.	Lac-F (GCAGCAGTAGGGAATCTTCCA) Lac-R (GCATTYCACCGCTACACATG)	[27]	Ct=−3.84x+34.93	0.999
Bifidobacterium spp.	BiTOT-F (TCGCGTCYGGTGTGAAAG) BiTOT-R (CCACATCCAGCRTCCAC)	[28]	Ct=−3.38x+41.06	0.995
Bacteroides fragilis group	Bfra-F (CGGAGGATCCGAGCGTTA) Bfra-R (CCGCAAACTTTCACAACTGACTTA)	[17]	Ct=−3.34x+40.48	0.989
Total Bacteria	F-Eub (ACTCCTACGGGAGGCAGCAG) R-Eub (ATTACCGCGGCTGCTGG)	[29]	Ct=−3.26x+40.18	0.999

Primers were synthesised by Eurofins (MWG, Ebersberg, Germany). Primer specificity was evaluated using the BLASTN algorithm [30] and specific amplification further confirmed experimentally by analysis of q-PCR melting curves.

Each 20 µl PCR amplification reaction contained 10 µl of Fast SYBR® Green Master Mix (Applied Biosystems), optimized concentrations of primers (Table S2), PCR grade water and 2 µl DNA (2.5 ng/µl). The reactions were performed in triplicate in a StepOne RealTime PCR System (Applied Biosystems, Foster City, CA) under the conditions given in Table S2.

Table S2: q-PCR programs, number cycles and primer concentrations used for the different bacterial group

Bacterial group	Initial denaturation	Denaturation	Annealing	Number of cycles	Fw (nM)	Rev (nM)
Lactobacillus spp.	95 °C – 20 s	95 °C – 3 s	63.5 °C – 30 s	40	200	200
Bifidobacterium spp.	95 °C – 20 s	95 °C – 3 s	60 °C – 35 s	40	200	300
B. fragilis group	95 °C – 20 s	95 °C – 3 s	60 °C – 30 s	40	300	300
Total Bacteria	95 °C – 10 m	95 °C – 20 s	53 °C – 20 s	40	250	250

The data obtained from the amplification were then divided by the average rRNA gene copy number for a particular genus or bacterial group and converted to obtain the number of bacteria expressed as Log CFU/g feces.[31,32] The rRNA copy number used to obtain the number of total bacteria/g feces was 4.34 which is an average of the rRNA gene copy number of the most representative phylum present in the newborn gut (Actinobacteria, Bacteroides, Proteobacteria and Firmicutes). Coefficients of determination (R^2) and the functions describing the relationship between Ct (threshold cycle) and x (log copy number) for the different assays are reported in Table S1.

Preparation of 16S V3 and V4 rRNA amplicons for Illumina MiSeq Pyrosequencing

Extracted DNA (stored at -80 °C) was processed to amplify and sequence the V3-V4 hypervariable region of the 16S rRNA gene.[33] These amplicons, approximately 550 bp in length, were generated using the forward primer = 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG - 3' and the reverse primer = 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'. 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). The PCR was performed using the following program: lid heated 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 and followed by a final elongation step at 72 °C for 5 min. PCR products were purified using the Agentcourt AMPure Kit (Beckman Coulter Genomics, United Kingdom).

Illumina sequencing adapters and dual-index barcodes were added to amplicons using the Nextera XT index kit (Illumina, San Diego, CA) according to manufacturer's instructions. The following program was utilised for 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. Amplicons were cleaned using the AMPure purification system and then quantified using Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, CA, USA). The quantified libraries were normalised and pooled to 4 nM, then the library was denatured with NaOH and further diluted with hybridization buffer prior to loading on a 2 x 300 nucleotide paired-end sequencing run on Illumina MiSeq platform at the Teagasc Food Research Centre.

Bioinformatic analysis

Raw sequence reads were assembled in to 300bp paired-end using FLASH.[34] Reads were further processed using the Qiime suite of tools, version 1.8.0,[35] including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds. Denoising, chimera detection, and operational taxonomic unit (OTU) grouping were performed using USEARCH v7.[36]

Taxonomic ranks were assigned to each sequence by alignment of OTUs using PyNAST [35] to the SILVA SSURef database, release 111. Alpha and beta diversities were generated in Qiime and calculated based on weighted and unweighted Unifrac distance matrices. Principal coordinate analysis (PCoA) plots were visualised using EMPeror v0.9.3-dev.[37]

Statistical analysis

MiniTab release 17 (MiniTab Ltd. Coventry, UK) was used to perform nonparametric statistical analysis. In order to examine significant differences between groups in the microbiota composition, (day 7 and day 30 of life, IAP treated and non-treated and breast-fed and mixed-fed infants), Mann-Whitney Test or Wilcoxon Signed Ranks Test were carried out respectively. Statistical significance of the microbial counts was defined as a P value of <0.05.

Results

Quantification of microbial groups in infant fecal samples via q-PCR

Total bacteria, bifidobacteria, lactobacilli and *B. fragilis* group members were quantified from fecal samples of all infants at day 7 and day 30 of life. The results are an average of Log CFU/g of feces and are shown in Figure 1.

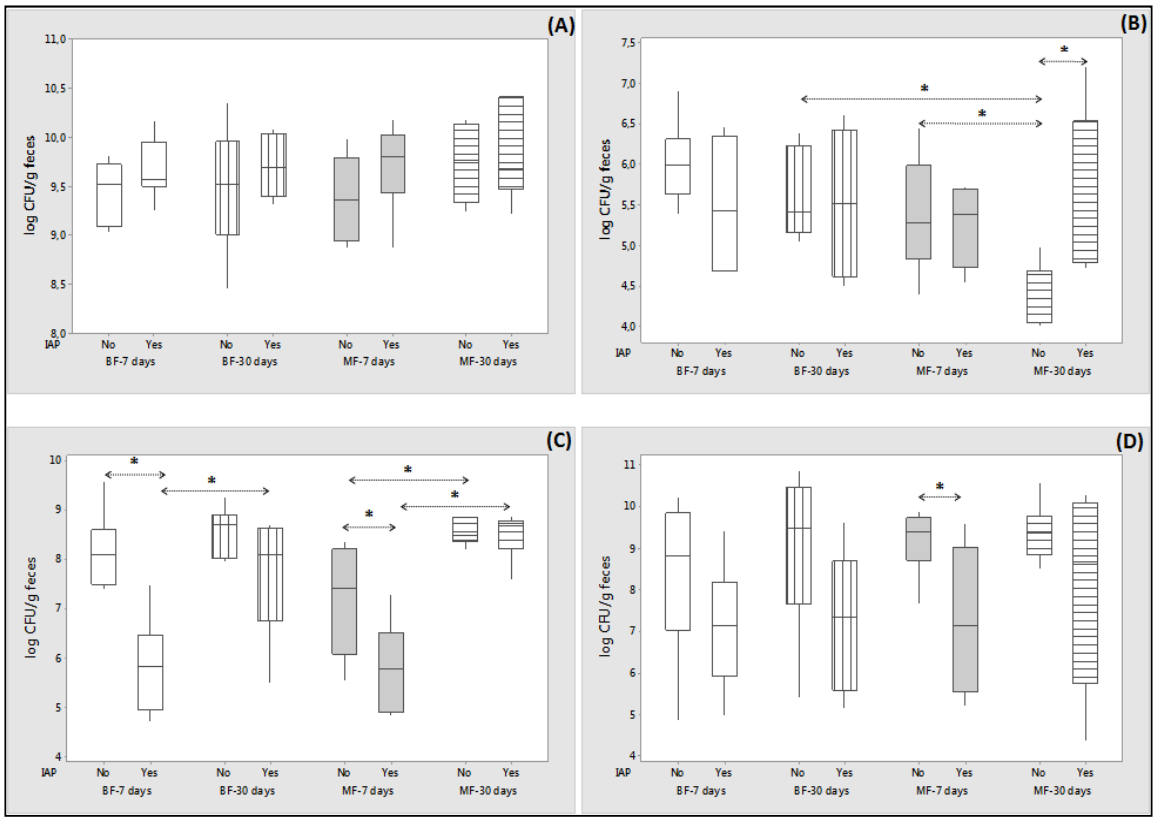

Figure 1: q-PCR analysis of selected microbial groups (**A**=Total bacteria; **B**=*Lactobacillus* spp.; **C**=*Bifidobacterium* spp.; **D**=*Bacteroides fragilis* group) from 51 samples separated by IAP treatment (data for control groups are indicated with No and IAP groups with Yes), feeding method and days of sampling: breast-fed (BF) at 7 days of life, n=6 and n=7, white boxes; breast-fed (BF) at 30 days of life, n=7 and n=7, white boxes with vertical lines; mixed-fed (MF) at 7 days of life, n=6 and n=6, grey boxes; mixed-fed (MF) at 30 days of life, n=6 and n=6, white boxes with horizontal lines. Horizontal line=median; asterisk=significant difference at P<0.05.

Levels of total bacteria ranged between 9.38 to 9.83 Log CFU/g of feces across samples. No significant differences between infants were observed, regardless of IAP treatment or mode of feeding (Figure 1A). Levels of total lactobacilli did not change significantly in the IAP treated groups over time, irrespective of mode of feeding. By contrast, in the control infants, those in the MF-C group sampled at day 30 had significantly lower lactobacilli than the MF-IAP group at day 30 ($P<0.013$), MF-C group at day 7 ($P<0.036$) and BF-C group at day 30 ($P<0.002$) (Figure 1B). Regarding *Bifidobacterium* spp., levels were observed to be significantly lower in infants belonging to IAP treated groups at day 7, both breast-fed ($P<0.005$) and mixed-fed ($P<0.03$) (Figure 1C), than in infants belonging to the control group. At 30 days of life, the difference in bifidobacterial counts between IAP treated groups and the respective controls was no longer significantly different. Irrespective of the feeding method (BF or MF), a significant increase was observed between the two sampling times in the IAP treated groups ($P<0,035$ and $P<0,036$, respectively). Furthermore, a significant increase of the *Bifidobacterium* spp. counts was observed in the MF-C group between the two sampling times ($P<0.028$) (Figure 1C).
Total *B. fragilis* levels were not significantly different between the BF-IAP and the BF-C groups, however at day 7 members of the *B. fragilis* group were detected in significantly lower levels in the MF-IAP infants than those in MF-C group ($P<0.045$) (Figure 1D).

Illumina MiSeq pyrosequencing of 16S rRNA amplicons from the fecal samples of IAP treated and control infants.

The V3-V4 region of bacterial 16S rRNA was amplified and sequenced on the MiSeq (Illumina) platform using DNA extracted from the infant fecal samples. A total of 9,731,890 quality-filtered sequences were obtained from these samples with an average of 200,000 reads per sample.
Diversity, richness, coverage and evenness estimations were calculated for all data sets (Figure 2). Statistical analysis using Mann-Whitney Test indicated that at day 7, the Shannon index and Simpson's index ($P<0.024$ and $P<0.014$, respectively; data not shown) were significantly lower in the IAP treated compared to the control infants (irrespective of feeding regime), no differences were reported at day 30. In particular, in breast-fed infants at day 7, statistically significant decreases in both diversity (Shannon) and richness (Simpson) indices ($P<0.007$ and $P<0.0154$, respectively) were observed in the IAP group compared with control infants (Figure 2).

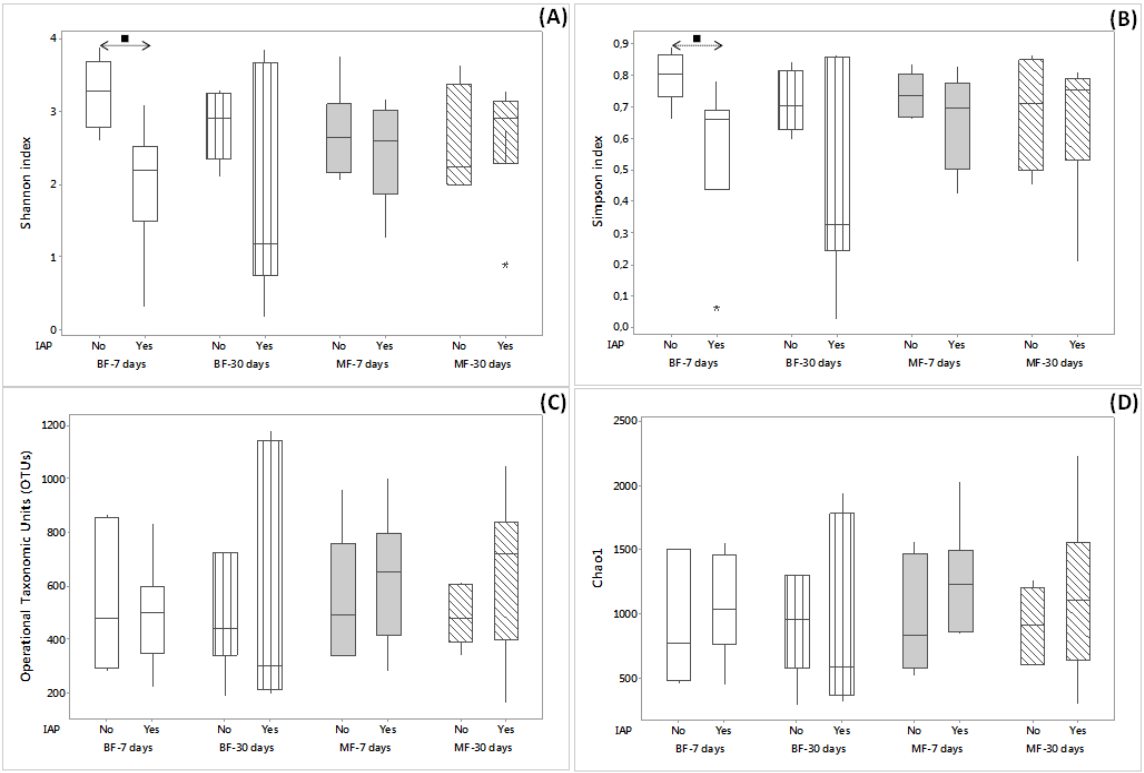

Figure 2: Richness and diversity indices relative to the different groups of infants separated by IAP treatment (data for control groups are indicated with No and IAP groups with Yes), feeding method and days of sampling: breast-fed (BF) at 7 days of life, n=6 and n=7, white boxes; breast-fed (BF) at 30 days of life, n=7 and n=7, white boxes with vertical lines; mixed-fed (MF) at 7 days of life, n=6 and n=6, grey boxes; mixed-fed (MF) at 30 days of life, n=6 and n=6, white boxes whit horizontal lines. Horizontal line=median; asterisk=outlier; filled square=significant difference at P<0.05. **A)** = Shannon index; **B)** = Simpson index; **C) =** operational taxonomic units (OTUs); **D)** = Chao1.

Composition of the gut microbiota over time (from 7 to 30 days of life).

Relative abundances of the dominant bacterial phyla, families and genera at day 7 and day 30 are shown in Table 2.

Table 2: Relative abundance of the most representative phyla, families and genera in faecal samples at 7 days and after 30 days of life.

	7 days n = 25			30 days n = 26		
Taxon	**Average**	**Min**	**Max**	**Average**	**Min**	**Max**
Firmicutes	37.5%	1.1%	99.1%	34.4%	10.5%	66.0%
Streptococcaceae	15.7%	0.0%	90.2%	6.1%	0.0%	34.1%
Streptococcus	15.6%	0.0%	90.2%	6.0%	0.0%	34.1%
Veillonellaceae	12.5%	0.0%	74.8%	21.3%	0.0%	59.9%
Veillonella	12.2%	0.0%	74.8%	18.5%	0.0%	59.8%
Clostridiaceae	2.3%	0.0%	30.1%	1.0%	0.0%	16.3%
Clostridium	2.3%	0.0%	30.1%	0.8%	0.0%	16.3%
Staphylococcaceae	2.2%	0.0%	32.7%	0.2%	0.0%	2.4%
Staphylococcus	2.0%	0.0%	32.7%	0.1%	0.0%	2.4%
Lactobacillaceae	1.9%	0.0%	44.5%	0.8%	0.0%	11.2%
Lactobacillus	1.9%	0.0%	44.5%	0.7%	0.0%	11.2%
Proteobacteria	31.5%	0.0%	98.6%	28.1%	0.0%	89.3%
Enterobacteriaceae	30.6%	0.0%	98.6%	27.3%	0.0%	89.3%
Escherichia	24.6%	0.0%	98.6%	20.0%	0.0%	86.4%
Bacteroidetes	22.1%	0.0%	81.1%	25.4%	0.0%	68.2%
Bacteroidaceae	17.3%	0.0%	72.1%	16.4%	0.0%	54.8%
Bacteroides	17.3%	0.0%	72.1%	16.3%	0.0%	54.8%
Porphyromonadaceae	4.7%	0.0%	41.6%	8.9%	0.0%	50.3%
Parabacteroides	4.7%	0.0%	41.6%	8.9%	0.0%	50.3%
Actinobacteria	6.2%	0.0%	65.5%	9.9%	0.0%	76.0%
Bifidobacteriaceae	5.5%	0.0%	63.9%	8.8%	0.0%	73.8%
Bifidobacterium	5.3%	0.0%	63.9%	8.7%	0.0%	73.7%
Verrucomicrobia	2.1%	0.0%	53.4%	1.8%	0.0%	48.0%
Verrucomicrobiaceae	2.1%	0.0%	53.4%	1.8%	0.0%	48.0%
Akkermansia	2.1%	0.0%	53.4%	1.8%	0.0%	47.9%

Additionally, aggregate taxonomical data at the phylum, family and genus level for each of the examined groups are shown in Figure 3, 4, 5.

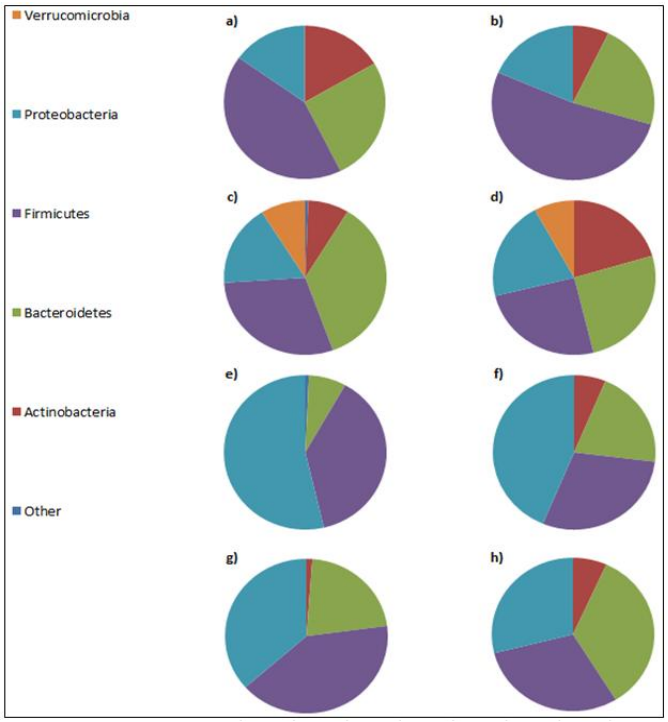

Figure 3: Aggregate taxonomical composition at phylum level in fecal samples from 25 newborns at 7 days of life: BF-C (panel a), MF-C (panel c); BF-IAP (panel e); MF-IAP (panel g) and from 26 newborns at 30 days of life: BF-C (panel b), MF-C (panel d); BF-IAP (panel f); MF-IAP (panel h).

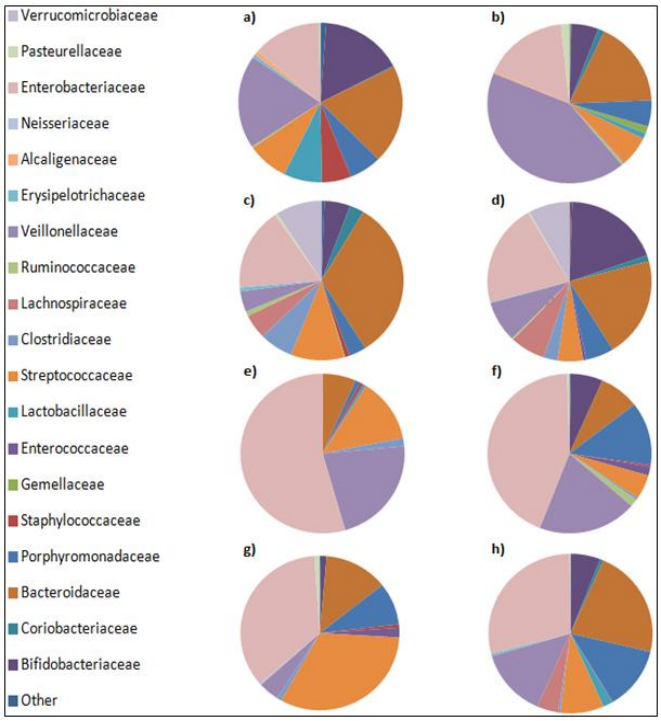

Figure 4: Aggregate taxonomical composition at family level in fecal samples from 25 newborns at 7 days of life: BF-C (panel a), MF-C (panel c); BF-IAP (panel e); MF-IAP (panel g) and from 26 newborns at 30 days of life: BF-C (panel b), MF-C (panel d); BF-IAP (panel f); MF-IAP (panel h).

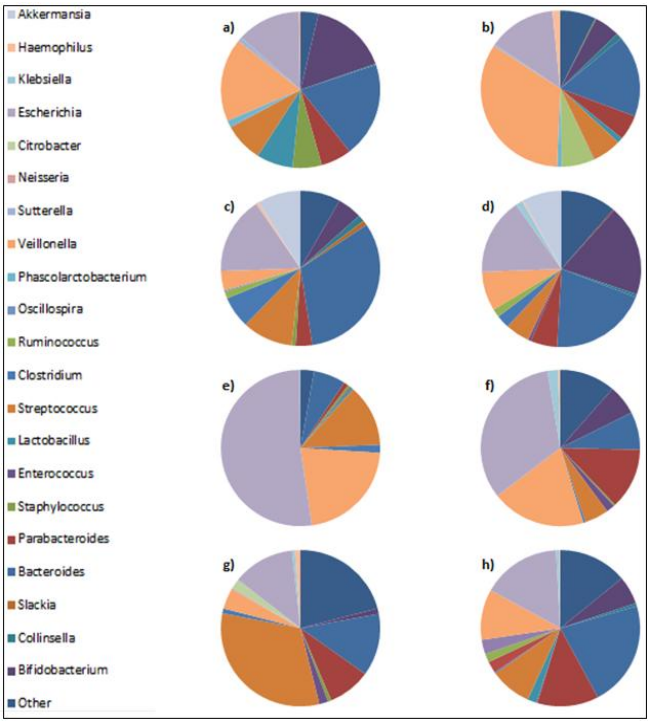

Figure 5: Aggregate taxonomical composition at genus level in fecal samples from 25 newborns at 7 days of life: BF-C (panel a), MF-C (panel c); BF-IAP (panel e); MF-IAP (panel g) and from 26 newborns at 30 days of life: BF-C (panel b), MF-C (panel d); BF-IAP (panel f); MF-IAP (panel h).

Taxonomy-based analysis showed that at the phylum level, more than 96% of the reads in all samples, both at day 7 and day 30, could be ascribed to four bacterial phyla, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria whose relative abundances varied widely from infant to infant (Table 2).

Only slight differences in the abundance of the phyla were found at day 7 and 30 indicating a relative stability over time. However, Firmicutes, Proteobacteria and Verrucomicrobia were more abundant at day 7 than at day 30, whereas Bacteroidetes and Actinobacteria were more represented after 30 days of life (Table 2).

Phylum Firmicutes included a number of families (*Streptococcaceae*, *Veillonellaceae*, *Clostridiaceae*, *Staphylococcaceae* and *Lactobacillaceae*). Among them, *Streptococcaceae* and *Veillonellaceae* were dominant at day 7 (15.7%) and day 30 (21.3%) (Table 2).

Within the Bacteroidetes, the families belonging to *Bacteroidaceae* and *Porphyromonadaceae* were represented, whereas Proteobacteria and Actinobacteria included only one family, *Enterobacteriaceae* and *Bifidobacteriaceae*, respectively (Table 2).

Notably, the phylum Verrucomicrobia was found only in one sample (MF-C) at day 7 (53.4%) and day 30 (48.0%) (Table 2). *Bifidobacterium* appeared to be more sensitive to antibiotic treatment than any other genus with a negligible relative abundance at day 7 and a strong growth

at day 30 (Figure 5 e,g,f,h). More specifically, taxonomy-based analysis on BF-IAP newborns showed a significantly higher abundance of the phylum Actinobacteria (Figure 3 e,f), of the family *Bifidobacteriaceae* (P<0.025) (Figure 4 e,f) and of the genus *Bifidobacterium* (P<0.025) at day 30 when compared to day 7 (Figure S1-A).

In the MF-IAP treated infants, there was a statistically significant higher proportion of reads corresponding to *Bifidobacterium* at day 30 (P<0.013) (Figure S1-B) and a trend towards significance for increased *Lachnospiraceae* (Firmicutes) (P<0.059) (Figure 4 g,h). Additionally, there was a significant decrease in *Staphylococcus* at day 30 in MF infants (P<0.042) (Figure S1-C). In the control infants, there was no significant difference in taxonomy between BF and MF over time.

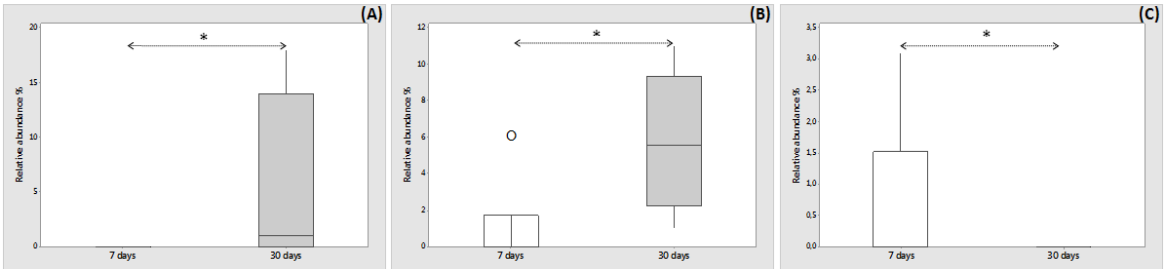

Figure S1: Significant difference in relative abundance of selected genera between IAP groups (**A**=*Bifidobacterium* in BF newborns; **B**=*Bifidobacterium* in MF newborns; **C**=*Staphylococcus* in MF newborns) at 7 days (white boxes) compared with 30 days (gray boxes). Horizontal line=median; circle=outlier; asterisk=significant difference at P<0.05.

Composition of the gut microbiota according to the type of treatment (IAP vs. Control).

At the phylum level, BF-IAP infants at day 7 showed significantly lower abundances of Actinobacteria (P<0.001), and a trend towards significance for higher abundances of Proteobacteria (P<0.062) compared to BF-C infants (Figure 3 a,e). Significant differences were observed between IAP and control infants at the family level: in BF-C infants at day 7 a statistically higher level of *Bifidobacteriaceae* (P<0.001) and lower level of *Enterobacteriaceae* (P<0.044) compared with BF-IAP was observed (Figure 4 a,e). Additionally, at day 30 there was a significant higher level of *Veillonellaceae* in BF-C infants compared with the BF-IAP group (P<0.035) (Figure 4 b,f).

Referring to the genus level, the gut microbiota of BF-C newborns at 7 days was dominated by genera belonging to the *Bacteroidaceae*, *Enterobacteriaceae* and *Bifidobacteriaceae* families (Figure 5 a), which accounted for approximately half of all genera detected at day 7 (Table 2). At day 7, the relative abundance of *Bifidobacterium* was significantly higher in BF-C than in BF-IAP infants (P<0.001) (Figure S2), in contrast, *Bacteroides* and *Escherichia* levels were similar

between C and IAP groups with a trend toward a significant increase in *Bacteroides* proportions and decrease in *Escherichia* in control group (P<0.078 and P<0.062, respectively) (Figure 5 a,e). When the relative abundance between IAP and C was examined in the MF infants, no significant differences were observed, both at day 7 and 30, although the family *Coriobacteriaceae* tended to be over represented in control infants compared with the IAP treated infants at day 7 (P<0.059) (Figure 4 c,g,d,h).

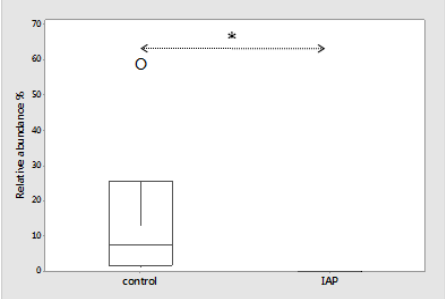

Figure S2: Significant difference in relative abundance of the genus *Bifidobacterium* in BF newborns at 7 days of life. Control, (white boxes), IAP newborns (gray boxes). Horizontal line=median; circle=outlier; asterisk=significant difference at P<0.05.

Composition of the gut microbiota by mode of feeding (BF vs. MF).

At day 7, when comparing feeding regime, no significant differences in microbial composition were detected both in IAP treated and control groups. A trend towards significance in an increase in *Clostridiaceae* and *Staphylococcus* in BF-C infants compared with MF-C (P<0.059) was observed (Figure 4 a,c and 5 a,c).

At day 30, BF-C had a significantly higher levels of Firmicutes (P<0.001), which accounted for 51.9% of all bacteria present, when compared with the MF infants (Figure 3 b,d). At the family level, a significantly higher number of *Lachnospiraceae* (P<0.042) was observed in MF-IAP compared with BF-IAP infants (Figure 4 h,f). Finally at genus level, in comparison with MF-IAP infants, BF-IAP had higher levels of *Escherichia* (P<0.040) (Figure S3).

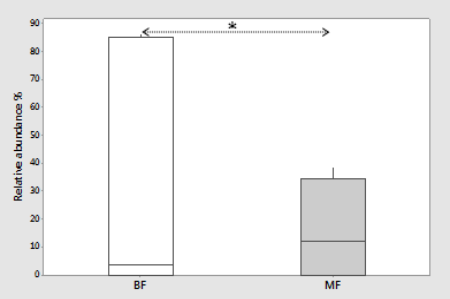

Figure S3: Significant difference in relative abundance of the genus *Escherichia* in IAP newborns at 30 days of life. BF (white boxes), MF newborns (gray boxes). Horizontal line=median; asterisk=significant difference at P<0.05.

Beta diversity

Principal coordinate analysis (PCoA) using unweighted Unifrac distances were utilised to examine the association of samples between different groups overtime (day 7 Vs. day 30), treatment (IAP treated Vs. control) and feeding type (BF Vs. MF) (Figure S4). No clustering was evident within the three groups of samples compared but separation between samples at day 7 and at day 30 (BF-C samples) (Figure S4-1B) and between control and IAP treated samples (BF at day 7) (Figure S4-2A) was observed.

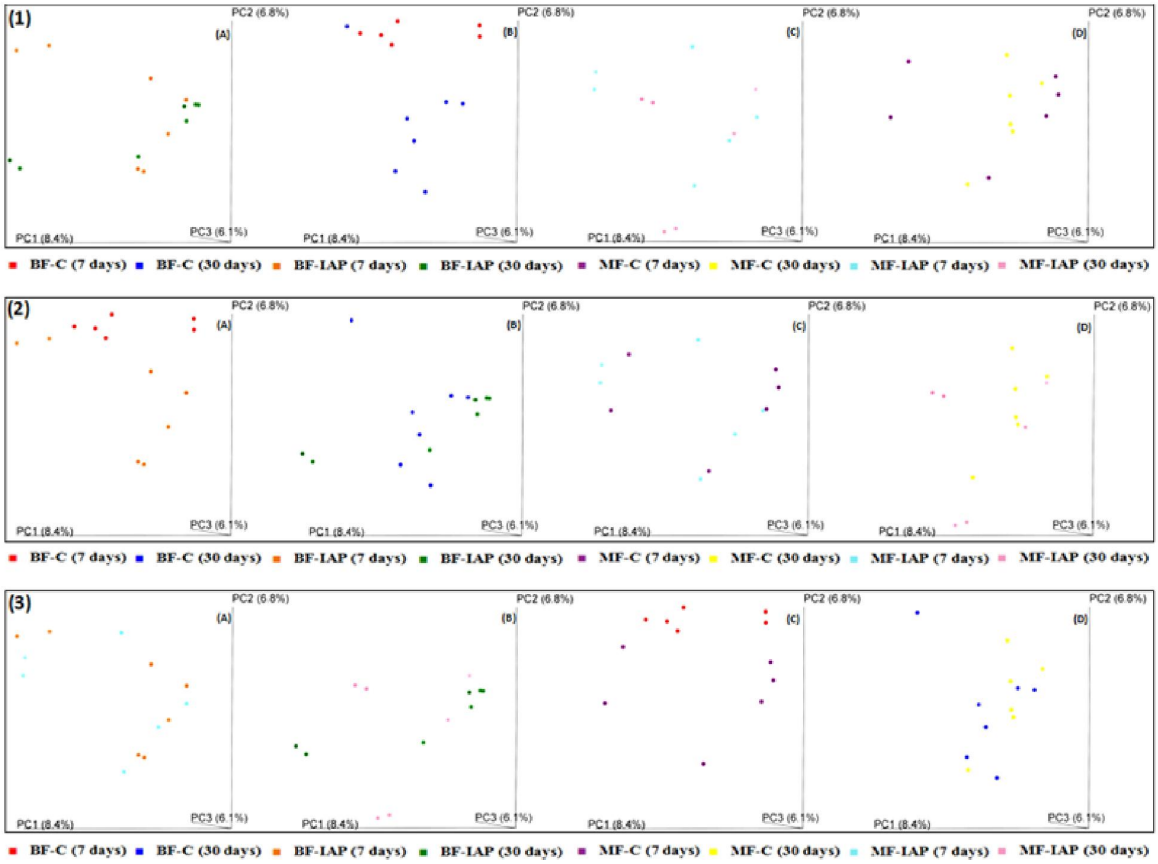

Figure S4: Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes on the scatterplot of the first three principal axes. Each point represents an individual subject.

(1)-PCoA in order to compare the time effect (7 days *Vs.* 30 days). (A) -Samples distribution in BF-IAP newborns. **(B)** -Samples distribution in BF-C newborns. **(C)** -Samples distribution in MF-IAP newborns. **(D)** -Samples distribution in MF-C newborns. **(2)-PCoA in order to compare the treatment impact (IAP *Vs.* C). (A)** -Samples distribution in BF newborns at 7 days. **(B)** -Samples distribution in BF newborns at 30 days. **(C)** -Samples distribution in MF newborns at 7 days of life. **(D)** -Samples distribution in MF newborns at 30 days. **(3)-PCoA in order to compare the feeding effect (BF *Vs.* MF). (A)** -Samples distribution in IAP newborns at 7 days. **(B)** -Samples distribution in IAP newborns at 30 days. **(C)** - Samples distribution in C newborns at 7 days. **(D)** -Samples distribution in C newborns at 30 days.

Discussions

A large number of papers and reviews have explored the complexity of microbial acquisition in the newborn gut during the first 48 months of life, as well as the factors which can affect gut microbial colonization such as mode of delivery, feeding type and antibiotic exposure.[17,23,38-40] Administration of antimicrobial agents, therapeutically or as prophylaxis to pregnant women, such as IAP to counteract early-onset GBS disease in infants causes alterations of maternal microbial population of vagina and intestinal tract.[41,42] Although the strong imprinting of the maternal microbiota on the early infant microbial population has been demonstrated,[9,16-18] only a few studies have focused on the effect of IAP in infants. A recent study[26] has shown that IAP treatment for GBS has a significant influence on the early bifidobacterial patterns, both qualitatively and quantitatively. However, a complete analysis of the effects of IAP on the overall bacterial population is lacking as well as information on the long term effects of IAP.

In the current study, next generation sequencing has been used for the first time to investigate the potential modulation of the newborn gut microbiota due to IAP. q-PCR was also used to achieve absolute quantification of targeted microbial genera. The study enrolled healthy infants, born at term, vaginally delivered, not treated with perinatal antibiotic and probiotics/prebiotics, breast-fed and mixed-fed infant, whose fecal material was sampled at day 7 and day 30 of life.

As already shown in other sequencing based infant studies, high variability in the inter-individual composition of gut microbiota has been observed in the present work.[43,44] Total bacterial counts were stable in all newborns throughout the trial period without significant difference between groups under investigation, in agreement with Hopkins et al.[45] who investigated the effect of different feeding types on the gut microbiota composition. Moreover, this study confirms an early reduction (at day 7 of life) of *Bifidobacterium* spp. counts in newborns whose mothers received IAP with respect to infants of control groups.[26] This difference is lower at 30 days, as shown both by q-PCR and sequencing analyses. In addition, high-throughput sequencing revealed an early reduction of microbial biodiversity in IAP infants, which was restored after one month. The dominant microbiota in the newborns studies was defined mainly by Firmicutes, Proteobacteria and Bacteroidetes, whose relative reads, on average, accounted for approximately 30% each. On the other hand, Actinobacteria were less represented with percentage values up to 10%. As already discussed in earlier studies,[45,46] this work confirmed using sequencing analyses, that the *Bacteroides* genus is highly represented in the newborn gut microbiota. Moreover, other dominant anaerobic genera of the early microbiota were *Bifidobacterium*, *Parabacteroides*, *Clostridium* and *Lactobacillus*. Sequencing results also

showed that the relative percentage of *Lactobacillus* was very low and significant differences were not detected between IAP and control groups. By contrast, real time results showed lower *Lactobacillus* counts in MF-C group with respect to MF-IAP at day 30. This observation does not have a specific explanation and should be confirmed by other studies.

The microbiota of the IAP newborns in the first week, compared with controls, was dominated by members of the *Enterobacteriaceae* family, which comprises potentially pathogenic strains. These observations are consistent with data obtained by Edwards et al.[47] who found that IAP selected ampicillin-resistant enterobacteria in the genital tract of the mother and, consequently, could increase the level of potential infectious bacteria in infants. In addition, Tanaka et al. [48] investigated the influence of antibiotic exposure during the postnatal period on the newborn gut microbiota development and found an inverse relation between the growth of *Enterobacteriaceae* and bifidobacteria.

In the present study we have considered the modulating effect of feeding type and its relation with IAP on gut colonization in infants. Numerous studies have demonstrated increased growth of beneficial bacteria belonging to the *Bifidobacterium* and *Lactobacillus* genera in the gut microbiota of breast-fed infants, whereas other authors have not found significant differences with regard to the type of feeding.[17,44,49-51] Sequencing results obtained in this work showed that feeding type did not influence the gut microbial colonization at 7 days, whereas several differences were observed after one month. The relative abundance of *Lactobacillus* did not change with the different feeding type, even though it was found that breast feeding had a significant positive impact on the levels of Firmicutes. No significant differences were also found in the Actinobacteria phylum, including the *Bifidobacterium* genus. Additionally, we found a greater effect of IAP in BF with respect to MF newborns. During the postnatal period, vertical bacterial transmission through breast milk has been reported in previous studies;[52,53] accordingly, it is possible to speculate a reduction of beneficial strains present in breast milk and, consequently, a reduced bacterial transmission from the mother to newborn.

In this study a DNA sequencing approach has been used for the first time to characterize the entire gut microbial population in newborns after intrapartum antibiotic treatment. The results, in combination with those acquired through q-PCR, confirmed and enriched those obtained in previous studies. The data indicate a reduction in biodiversity and richness in IAP groups compared with controls, definitively demonstrating the negative and short term consequence of IAP in early gut bacterial population, the impact being higher for breast fed newborns. The bacteria most affected by antibiotic treatment belonged to the *Bifidobacterium* genus followed by, to Bacteroides. Most of the differences were equalized at 30 days, when the gut colonization

reaches a higher stability. These results open the perspective of investigating the IAP impact on vertical bacterial transmission, mother to newborn, during delivery. The analyses of microbiota from vagina, breast milk and gut of pregnant women and gut microbiota of matched newborns could allow to assess the impact of IAP at different levels and to provide new insights in order to limit the negative effect of IAP as well as to define new strategies to control GBS infection in pregnant women.

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Author contribution

All authors contributed extensively to the work presented in this paper. G. Mazzola and K. Murphy designed and performed experiments, analysed data and drafted the paper; D. Di Gioia, C. Stanton, R. P. Ross supervised the project and the writing of the paper; L.T. Corvaglia and G. Faldella recruited participants and obtained informed written consent.

References:

1 Bromberger P, Lawrence JM, Braun D *et al*. The influence of intrapartum antibiotics on the clinical spectrum of early-onset group B streptococcal infection in term infants. *Pediatrics* 2000; **106**: 244–50.

2 Edwards MS, Nizet V. Group B streptococcal infections. In: Remington JS, Klein JO, Wilson CB *et al.*, ed. *Diseases of the fetus and newborn infant*. Philadelphia: Elsevier, 2011; 419–69.

3 Barcaite E, Bartusevicius A, Tameliene R *et al*. Prevalence of maternal group B streptococcal colonisation in European countries. *Acta Obstet Gynecol Scand* 2008; **87**: 260–71.

4 Stoll BJ, Hansen NI, Sánchez PJ *et al*. Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues. *Pediatrics* 2011; **127**: 817–26.

5 Yagupsky P, Menegus M, Powell K. The changing spectrum of group B streptococcal disease in infants: an eleven-year experience in a tertiary care hospital. *Pediatr Infect Dis J* 1991; **10**: 801–8.

6 Garland SM. Early Onset Neonatal Group B Streptococcus (GBS) Infection: Associated Obstetric Risk Factors. *Aust New Zeal J Obstet Gynaecol* 1991; **31**: 117–8.

7 Oddie S. Risk factors for early onset neonatal group B streptococcal sepsis: case-control study. *BMJ* 2002; **325**: 308–308.

8 Centers for Disease Control and Prevention. *Prevention of group B streptococcal disease: a public health perspective*. *Mor Mortal Wkly Rep* 45: 1–24, 1996.

9 Verani J, McGee L, Schrag S *et al*. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC, 2010. *Mor Mortal Wkly Rep* 2010; **59**: 1–32.

10 Van Dyke MK, Phares CR, Lynfield R *et al*. Evaluation of universal antenatal screening for group B streptococcus. *N Engl J Med* 2009; **360**: 2626–36.

11 Schrag S, Gorwitz R, Fultz-Butts K *et al*. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. *Morb Mortal Wkly Rep Recomm Rep* 2002; **51**: 1–22.

12 Lin FY, Brenner RA, Johnson YR *et al*. The effectiveness of risk-based intrapartum chemoprophylaxis for the prevention of early-onset neonatal group B streptococcal disease. *Am J Obstet Gynecol* 2001; **184**: 1204–10.

13 Dominguez-Bello MG, Costello EK, Contreras M *et al*. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA* 2010; **107**: 11971–5.

14 Kelly D, King T, Aminov R. Importance of microbial colonization of the gut in early life to the development of immunity. *Mutat Res* 2007; **622**: 58–69.

15 Morelli L. Postnatal development of intestinal microflora as influenced by infant nutrition. *J Nutr* 2008; **138**: 1791S–5S.

16 Palmer C, Bik EM, DiGiulio DB *et al*. Development of the human infant intestinal microbiota. *PLoS Biol* 2007; **5**: e177.

17 Penders J, Thijs C, Vink C *et al*. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* 2006; **118**: 511–21.

18 Adlerberth I, Wold a E. Establishment of the gut microbiota in Western infants. *Acta Paediatr* 2009; **98**: 229–38.

19 O'Toole PW, Claesson MJ. Gut microbiota: Changes throughout the lifespan from infancy to elderly. *Int Dairy J* 2010; **20**: 281–91.

20 Hällström M, Eerola E, Vuento R *et al*. Effects of mode of delivery and necrotising enterocolitis on the intestinal microflora in preterm infants. *Eur J Clin Microbiol Infect Dis* 2004; **23**: 463–70.

21 Westerbeek EAM, van den Berg A, Lafeber HN *et al*. The intestinal bacterial colonisation in preterm infants: a review of the literature. *Clin Nutr* 2006; **25**: 361–8.

22 Penders J, Thijs C, van den Brandt P a *et al*. Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study. *Gut* 2007; **56**: 661–7.

23 Marques TM, Wall R, Ross RP *et al*. Programming infant gut microbiota: influence of dietary and environmental factors. *Curr Opin Biotechnol* 2010; **21**: 149–56.

24 Sazawal S, Dhingra U, Hiremath G *et al*. Prebiotic and probiotic fortified milk in prevention of morbidities among children: community-based, randomized, double-blind, controlled trial. *PLoS One* 2010; **5**: e12164.

25 Keski-Nisula L, Kyynäräinen H-R, Kärkkäinen U *et al*. Maternal intrapartum antibiotics and decreased vertical transmission of *Lactobacillus* to neonates during birth. *Acta Paediatr* 2013; **102**: 480–5.

26 Aloisio I, Mazzola G, Corvaglia LT *et al*. Influence of intrapartum antibiotic prophylaxis against group B *Streptococcus* on the early newborn gut composition and evaluation of the anti-*Streptococcus* activity of *Bifidobacterium* strains. *Appl Microbiol Biotechnol* 2014; **98**: 6051–60.

27 Castillo M, Martín-Orúe SM, Manzanilla EG *et al*. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet Microbiol* 2006; **114**: 165–70.

28 Rinttilä T, Kassinen A, Malinen E *et al*. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004; **97**: 1166–77.

29 Guo X, Xia X, Tang R *et al*. Real-time PCR quantification of the predominant bacterial divisions in the distal gut of Meishan and Landrace pigs. *Anaerobe* 2008; **14**: 224–8.

30 Altschul SF, Madden TL, Schäffer AA *et al*. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**: 3389–402.

31 Klappenbach JA, Saxman PR, Cole JR *et al*. rrndb: the Ribosomal RNA Operon Copy Number Database. *Nucleic Acids Res* 2001; **29**: 181–4.

32 Lee ZM-P, Bussema C, Schmidt TM. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Res* 2009; **37**: D489–93.

33 Klindworth A, Pruesse E, Schweer T *et al*. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 2013; **41**: e1.

34 Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 2011; **27**: 2957–63.

35 Caporaso JG, Kuczynski J, Stombaugh J *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; **7**: 335–6.

36 Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010; **26**: 2460–1.

37 Vázquez-Baeza Y, Pirrung M, Gonzalez A *et al*. EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* 2013; **2**: 16.

38 Di Gioia D, Aloisio I, Mazzola G *et al*. Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Appl Microbiol Biotechnol* 2014; **98**: 563–77.

39 Power SE, O'Toole PW, Stanton C *et al*. Intestinal microbiota, diet and health. *Br J Nutr* 2014; **111**: 387–402.

40 Scheepers LEJM, Penders J, Mbakwa CA *et al*. The intestinal microbiota composition and weight development in children: the koala birth cohort study. *Int J Obes (Lond)* 2014.

41 Bizzarro MJ, Dembry L-M, Baltimore RS *et al*. Changing patterns in neonatal *Escherichia coli* sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. *Pediatrics* 2008; **121**: 689–96.

42 Sullivan A, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis* 2001; **1**: 101–14.

43 Abrahamsson TR, Jakobsson HE, Andersson AF *et al*. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012; **129**: 434–40, 440.e1–2.

44 Azad MB, Konya T, Maughan H *et al*. Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. CMAJ 2013; **185**: 385–94.

45 Hopkins MJ, Macfarlane GT, Furrie E *et al*. Characterisation of intestinal bacteria in infant stools using real-time PCR and northern hybridisation analyses. *FEMS Microbiol Ecol* 2005; **54**: 77–85.

46 Wang M, Ahrné S, Antonsson M *et al*. T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. *J Microbiol Methods* 2004; **59**: 53–69.

47 Edwards RK, Clark P, Sistrom CL *et al*. Intrapartum antibiotic prophylaxis 1: relative effects of recommended antibiotics on gram-negative pathogens. *Obstet Gynecol* 2002; **100**: 534–9.

48 Tanaka S, Kobayashi T, Songjinda P *et al*. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunol Med Microbiol* 2009; **56**: 80–7.

49 Solís G, de Los Reyes-Gavilan CG, Fernández N *et al*. Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast-milk and the infant gut. *Anaerobe* 2010; **16**: 307–10.

50 Bezirtzoglou E, Stavropoulou E. Immunology and probiotic impact of the newborn and young children intestinal microflora. *Anaerobe* 2011; **17**: 369–74.

51 Fan W, Huo G, Li X *et al*. Diversity of the intestinal microbiota in different patterns of feeding infants by Illumina high-throughput sequencing. *World J Microbiol Biotechnol* 2013; **29**: 2365–72.

52 Jost T, Lacroix C, Braegger CP *et al*. New insights in gut microbiota establishment in healthy breast fed neonates. *PLoS One* 2012; **7**: e44595.

53 Perez PF, Doré J, Leclerc M *et al*. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics* 2007; **119**: e724–32.

General discussion and conclusions

The work reported in this thesis was aimed to study factors affecting the gut microbial colonization in the early stage of life and to suggest possible strategies to maintain or restore a correct microbial gut balance in infants. In order to explain and understand the role of the genus *Bifidobacterium* within the gut microbiota and its relationship towards the health host's status, this study encloses different closely-related works.

The first goal of this Ph.D thesis was to review the current literature regarding to the probiotics and prebiotics application in infants with a particular attention to bifidobacteria. Currently, the research on this topic is particularly active. It underlines the efficacy of some probiotic strains for the treatment of targeted diseases and stresses that newborns and children possessing an unstable gut microbial composition are more susceptible to variations caused by external factors. In this respect, bifidobacteria play a pivotal role and their use in pediatrics as preventive or therapeutic agents is an established fact, both for enteric diseases and diseases which are not apparently linked to the gastro-intestinal tract. Moreover, several papers agree to enrich the formula milk with prebiotics to make the gut microbial composition of formula fed infants more similar to that of breast-fed newborns. These up to date on probiotics and prebiotics application led to the preparation of a book's chapter and a review, respectively:

1) "Infant development, currently the main applications of probiotics and prebiotics?" Mazzola G et al., (2015) within the book "Probiotics and Prebiotics: Current Research and Future Trends"

2) "Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants" Di Gioia D et al., (2014), Applied Microbiology and Biotechnology

The second intent of this thesis was to evaluate the potential applications in infants of selected bifidobacteria also in combination with prebiotic fibers. Within this aim, the antimicrobial activity of previously selected *Bifidobacterium* strains from infant feces (Aloisio et al., 2012) against potentially pathogenic bacteria of infants was assessed. For this purpose continuous culture fermentation simulating the gut microbiota of a 2-month-old colicky infant were performed and challenged with the strain *B. breve* B632 (previously selected in the study of Aloisio et al., 2012). To examine the time-course of *E. coli*, *Enterobacteriaceae* and *Bifidobacterium* spp. populations, fluorescent in situ hybridization (FISH) and quantitative PCR

(q-PCR) were performed, while the random amplification of polymorphic DNA (RAPD-PCR) was applied to trace the strain of *B. breve* B632 inoculated in the fecal cultures.

In addition, commercial fibers were screened for their selective stimulation towards bifidobacteria strains. Last but not least, resistance of selected strains to simulated gastro-intestinal conditions was evaluated. The results have globally shown that the strain *B. breve* B632, as lyophilized strain or in a microencapsulated form, is a suitable candidate, compared to the other bifidobacteria strains assayed, for the use as probiotic in infants. Additionally, the selection of commercial fibers allowed the identification of two oligosaccharides, a fructooligosaccharide with a DP lower than 10 (Orafti®HSI) and a galactooligosaccharide (Vivinal®) which were able to stimulate selectively the growth of the *B. breve* B632. The same strain of *B. breve* was able to survive in a complex microbial environment when it was inoculated within gut microbiota cultures of a colicky newborn, simulating *in vivo* conditions, as well as to exert antimicrobial activity against *Enterobacteriaceae*.

Thanks to the present study, which complements already published results (Aloisio et al., 2012), the *B. breve* B632 strain is one of the component of a probiotic formulation targeted to newborns for the prevention/treatment of colics. The next goal is the formulation of a synbiotic product for the re-establishment of the correct microbial balance in newborns after disbiosis.

This work led to the preparation of two papers:

3) "The Probiotic *Bifidobacterium breve* B632 Inhibited the Growth of *Enterobacteriaceae* within Colicky Infant Microbiota Cultures" Simone M et al., (2014), BioMed Research International

4) "Development of a synbiotic product for newborns and infants" Giuseppe M et al., (2015), under submission

Several factors (e.g., feeding type, use of antibiotics during early stages of life, gestational age at birth, hospitalization after birth, mode of delivery) in early life can lead to a perturbation in the development of the gut microbiota. The impact of intrapartum antibiotic prophylaxis (IAP), against group B *Streptococcus* (GBS), on the newborn gut colonization has been studied for the first time in this work.

Healthy, born at term, vaginally delivered, not treated with perinatal antibiotic and probiotics/prebiotics infants were enrolled. The microbial composition was assessed using a

combination of culture independent techniques which included q-PCR, denaturing gradient gel electrophoresis (DGGE) and the high-throughput pyrosequencing of 16S rRNA.

In the first study 52 fecal DNA from infants, exclusively breast-fed, at 7 days of life were analyzed. The evaluation by q-PCR of the main microbial groups in infant fecal samples, *Lactobacillus* spp., *Bidobacterium* spp., *Bacteroides fragilis* group, *C. difficile* and *E. coli*, showed a significant reduction of the bifidobacteria counts following IAP. DGGE approach, targeted to evaluate the biodiversity within the bifidobacteria population, showed that IAP determined a strong decrement in the frequency of some *Bifidobacterium* species such as *B. breve*, *B. bifidum* and *B. dentium*. Additionally, this work showed that selected bifidobacteria strains (see papers 3 and 4) exerted, *in vitro*, an antimicrobial activity towards *S. agalactiae* strains isolated from positive-GBS women. These results provided preliminary evidences on the possibility of using a non-chemotherapeutic approach for the prevention of GBS infections, i.e., the administration of selected bifidobacteria in the positive pregnant women to GBS.
This work led to the preparation of one paper:

5) "Influence of intrapartum antibiotic prophylaxis against group B *Streptococcus* on the early newborn gut composition and evaluation of the anti-*Streptococcus* activity of *Bifidobacterium* strains" Aloisio I et al., (2014), Applied Microbiology and Biotechnology

The second part of this study was aimed to shed light on the short term effects of IAP and also to its effect at one month of life. Additionally, two types of feeding were examined.
A total of 84 subjects were enrolled and followed up over one month of life and clustered into two groups according to the feeding type: exclusively human breast-fed or formula-fed. Selected microbial groups, *Lactobacillus* spp., *Bifidobacterium* spp. and *Bacteroides fragilis* were monitored via q-PCR from DNA extracted from fecal samples. As already shown in the previous study the fecal counts of *Bifidobacteria* were reduced by maternal IAP in the first week of life but they get back to normal after one month of life. Thus, the first achievement of this study was that IAP in GBS-positive mothers had an early and transient influence on the infant's gut microbiota by reducing the count of *Bifidobacteria* which was recovered after one month. Furthermore, the use of formula milk represented an additional negative factor in terms of bifidobacteria colonization. The other microbial groups examined did not show any significant variations both at 7 days and at 30 days of life.

This work led to the preparation of one paper:

6) "Influence of intrapartum antibiotic prophylaxis for Group B *Streptococcus* and type of feeding on gut microbiota during the first month of life" Corvaglia L et al., (2015), under submission

While the absolute quantification of selected bacterial groups is important, the groups examined represent only a small proportion of the overall intestinal microbiota. Moreover, a complete analysis of the effects of IAP on the overall gut bacterial population in infants was lacking.
In this study a DNA high-throughput pyrosequencing approach (Illumina MiSeq System), in combination with the q-PCR analysis, was used for the first time to enrich and better understand the IAP impact at 7 days and at 30 days of life. Moreover, a comparison of the gut composition between breast-fed and mixed-fed infants (breast-fed plus at least 50% of formula milk), was also performed at the same sampling times.
Sequencing results (performed at the Food Biosciences Department, Teagasc Food Research Centre, Fermoy, Co. Cork, IE) indicated a reduction in biodiversity and richness observed in IAP group and definitively demonstrated the negative and short term consequence of IAP on early gut bacterial population. The bacteria which resulted more affected by antibiotic treatment belonged to the *Bifidobacterium* genus and, secondly, to the *Bacteroides* one. The microbiota of the IAP newborns in the first week, compared to not-IAP controls, was dominated by members of the *Enterobacteriaceae* family, which comprise potentially pathogenic strains. Additionally the feeding type did not influence the gut microbial colonization at 7 days, whereas several differences were observed at one month. In particular, at this time point, the breast-fed control infants had a significantly higher levels of Firmicutes ($P<0.001$), and the breast-fed IAP treated infants had higher levels of *Escherichia* ($P<0.040$) compared to the mixed-fed infants. The relative abundance of *Lactobacillus* did not change with the different feeding types, even though it was found that breast feeding had a significantly positive impact on levels of Firmicutes. Furthermore, this work reported a greater effect of IAP in breast-fed infants compared to infants feeding with a mixture of breast and formula milk which can be explained by a possible reduction of beneficial bacteria present in breast milk and consequently a reduced bacterial transmission from the mother to newborn.

This work led to the preparation of one paper:

7) "High-throughput sequencing and q-PCR approach to study early gut microbiota perturbations following intrapartum antibiotic prophylaxis to prevent group B streptococcal disease" Mazzola G et al., (2015), under submission

Therefore, the main achievements reached in this dissertation are that the strain *B. breve* B632, as lyophilized strain or in a microencapsulated form, is a possible candidate to offset the problems which gas-producing coliforms or streptococcal infections cause to the infant's gut microbiota. In order to strengthen the probiotic effect and to stimulate selectively the growth of the strain B632, it has been hypothesized its use in a synbiotic product coupled to a mixture of selected prebiotic fibers, a galactooligosaccharide and a fructooligosaccharide. A clinical studies on newborns to which a probiotic preparation containing this strain has been administered is at present on-going to check the efficacy of *Bifidobacterium* spp. administration on the prevention of colics in infants.

The investigation of the early gut microbial composition has allowed to expand scientific knowledge about the factors that contribute to the development of the gut microbiota in newborns. These studies have shown that IAP could affect the early intestinal balance in infants and they have paved the way to the definition of new strategies alternative to antibiotic treatment to control GBS infection in pregnant women.

References

Adlerberth I, Wold a E (2009) Establishment of the gut microbiota in Western infants. Acta Paediatr 98:229–38.

Allen J, Hector D (2005) Benefits of breastfeeding. N S W Public Health Bull 16:42.

Aloisio I, Santini C, Biavati B, Dinelli G, Cencič A, Chingwaru W, Mogna L, Di Gioia D (2012) Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns. Appl Microbiol Biotechnol 96:1561–76.

Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F (2011) Diarrheagenic *Escherichia coli* in acute gastroenteritis in infants in North-West Italy. New Microbiol 34:45–51.

Arboleya S, Ruas-Madiedo P, Margolles A, Solís G, Salminen S, de Los Reyes-Gavilán CG, Gueimonde M (2011) Characterization and in vitro properties of potentially probiotic *Bifidobacterium* strains isolated from breast-milk. Int J Food Microbiol 149:28–36.

Arboleya S, Binetti A, Salazar N, Fernández N, Solís G, Hernández-Barranco A, Margolles A, de Los Reyes-Gavilán CG, Gueimonde M (2012) Establishment and development of intestinal microbiota in preterm neonates. FEMS Microbiol Ecol 79:763–72.

Azad MB, Konya T, Maughan H, Guttman DS, Field CJ, Chari RS, Sears MR, Becker AB, Scott JA, Kozyrskyj AL (2013) Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. CMAJ 185:385–94.

Bäckhed F, Ding H, Wang T, Hooper L V, Koh GY, Nagy A, Semenkovich CF, Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 101:15718–23.

Barrett E, Kerr C, Murphy K, O'Sullivan O, Ryan CA, Dempsey EM, Murphy BP, O'Toole PW, Cotter PD, Fitzgerald GF, Ross RP, Stanton C (2013) The individual-specific and diverse nature of the preterm infant microbiota. Arch Dis Child Fetal Neonatal Ed 98:F334–40.

Begley M, Gahan CGM, Hill C (2005) The interaction between bacteria and bile. FEMS Microbiol Rev 29:625–51.

Begley M, Hill C, Gahan CGM (2006) Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72:1729–38.

Ben X-M, Li J, Feng Z-T, Shi S-Y, Lu Y-D, Chen R, Zhou X-Y (2008) Low level of galacto-oligosaccharide in infant formula stimulates growth of intestinal Bifidobacteria and Lactobacilli. World J Gastroenterol 14:6564–8.

Bennet R, Eriksson M, Nord CE (2002) The Fecal Microflora of 1–3-Month-Old Infants during Treatment with Eight Oral Antibiotics. Infection 30:158–160.

Benno Y, Sawada K, Mitsuoka T (1984) The Intestinal Microflora of Infants: Composition of Fecal Flora in Breast-Fed and Bottle-Fed Infants. Microbiol Immunol 28:975–986.

Bergman NJ (2013) Neonatal stomach volume and physiology suggest feeding at 1-h intervals. Acta Paediatr 102:773–7.

Bezirtzoglou E, Stavropoulou E (2011) Immunology and probiotic impact of the newborn and young children intestinal microflora. Anaerobe 17:369–74.

Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C (2010) Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev 86 Suppl 1:13–5.

Biavati B, Castagnoli P, Trovatelli LD (1986) Species of the genus *Bifidobacterium* in the feces of human adults. Microbiologica 9:39–45.

Biavati B, Mattarelli P (2012) Genus Bifidobacterium, in: Goodfellow M, Kampfer P, Busse H J, Suzuki KI, WL & WBW (Ed), Bergey's Manual of Systematic Bacteriology pp. 171-206.

Bin-Nun A, Bromiker R, Wilschanski M, Kaplan M, Rudensky B, Caplan M, Hammerman C (2005) Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. J Pediatr 147:192–6.

Bode L (2009) Human milk oligosaccharides: prebiotics and beyond. Nutr Rev 67 Suppl 2:S183–91.

Boehm G, Moro G (2008) Structural and functional aspects of prebiotics used in infant nutrition. J Nutr. 138:1818-1828.

Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, White J-SS (2009) Generalized linear mixed models: a practical guide for ecology and evolution. Trends Ecol Evol 24:127–135.

Borruel N (2002) Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. Gut 51:659–664.

Bottacini F, Medini D, Pavesi A, Turroni F, Foroni E, Riley D, Giubellini V, Tettelin H, van Sinderen D, Ventura M (2010) Comparative genomics of the genus *Bifidobacterium*. Microbiology 156:3243–54.

Braga TD, Alves G, Israel P, Lira C De, Lima MDC (2011) Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind , randomized , controlled trial 1–3. 81–86.

Brandtzaeg P, Pabst R (2004) Let's go mucosal: communication on slippery ground. Trends Immunol 25:570–7.

Burgain J, Gaiani C, Linder M, Scher J (2011) Encapsulation of probiotic living cells: From laboratory scale to industrial applications. J Food Eng 104:467–483.

Burns P, Sánchez B, Vinderola G, Ruas-Madiedo P, Ruiz L, Margolles A, Reinheimer J, de los Reyes-Gavilán CG (2010) Inside the adaptation process of *Lactobacillus delbrueckii* subsp. *lactis* to bile. Int J Food Microbiol 142:132–41.

Camilo E, Zimmerman J, Mason J, Golner B, Russell R, Selhub J, Rosenberg I (1996) Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. Gastroenterology 110:991–998.

Campbell JM, Fahey GC, Wolf BW (1997) Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. J Nutr 127:130–6.

Caplan MS (2009) Probiotic and prebiotic supplementation for the prevention of neonatal necrotizing enterocolitis. J Perinatol 29 Suppl 2:S2–6.

Cencic A, Langerholc T (2010) Functional cell models of the gut and their applications in food microbiology--a review. Int J Food Microbiol 141 Suppl :4–14.

Cerutti A, Rescigno M (2008) The biology of intestinal immunoglobulin A responses. Immunity 28:740–50.

Champagne CP, Ross RP, Saarela M, Hansen KF, Charalampopoulos D (2011) Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. Int J Food Microbiol 149:185–93.

Chonan O, Takahashi R, Watanuki M (2001) Role of activity of gastrointestinal microflora in absorption of calcium and magnesium in rats fed beta1-4 linked galactooligosaccharides. Biosci Biotechnol Biochem 65:1872–5.

Cirgin Ellett ML (2003) What Is Known About Infant Colic? Gastroenterol Nurs 26:60-5.

Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW (2011) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A 108 Suppl :4586–91.

Claud EC (2001) Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. FASEB J 15:1398–1403.

Comalada M, Bailón E, de Haro O, Lara-Villoslada F, Xaus J, Zarzuelo A, Gálvez J (2006) The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. J Cancer Res Clin Oncol 132:487–97.

Coppa G V, Zampini L, Galeazzi T, Gabrielli O (2006) Prebiotics in human milk: a review. Dig Liver Dis 38 Suppl 2:S291–4.

Coppa G, Gabrielli O, (2008) Human milk oligosaccharides as prebiotics. In: Versalovic J and Wilson M (eds.) Therapeutic microbiology: probiotics and related strategies. American Society for Microbiology Press, Washington, pp. 131–146.

Corrêa NB, Péret Filho LA, Penna FJ, Lima FM, Nicoli JR (2005) A randomized formula controlled trial of *Bifidobacterium lactis* and *Streptococcus thermophilus* for prevention of antibiotic-associated diarrhea in infants. J Clin Gastroenterol 39:385-9.

Cryan JF, Dinan TG (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13:701–12.

Da Cruz AG, Faria J de AF, Van Dender AGF (2007) Packaging system and probiotic dairy foods. Food Res Int 40:951–956.

De Weerth C, Fuentes S, Puylaert P, de Vos WM (2013) Intestinal microbiota of infants with colic: development and specific signatures. Pediatrics 131:e550–8.

Del Piano M, Carmagnola S, Ballarè M, Sartori M, Orsello M, Balzarini M, Pagliarulo M, Tari R, Anderloni A, Strozzi GP, Mogna L, Sforza F, Capurso L (2011) Is microencapsulation the future of probiotic preparations? The increased efficacy of gastro-protected probiotics. Gut Microbes 2:120–123.

Delattre C, Vijayalakshmi MA (2009) Monolith enzymatic microreactor at the frontier of glycomic toward a new route for the production of bioactive oligosaccharides. J Mol Catal B Enzym 60:97–105.

Delzenne NM, Neyrinck AM, Bäckhed F, Cani PD (2011) Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 7:639–46.

Deshpande G, Rao S, Patole S, Bulsara M (2010) Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics 125:921–30.

DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, Kim CJ, Erez O, Edwin S, Relman D (2008) Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PLoS One 3:e3056.

Dogi CA, Galdeano CM, Perdigón G (2008) Gut immune stimulation by non pathogenic Gram(+) and Gram(-) bacteria. Comparison with a probiotic strain. Cytokine 41:223–31.

Dong X, Xin Y, Jian W, Liu X, Ling D (2000) *Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester. Int J Syst Evol Microbiol 50:119–125.

Duerr CU, Hornef MW (2012) The mammalian intestinal epithelium as integral player in the establishment and maintenance of host-microbial homeostasis. Semin Immunol 24:25–35.

Edelson MB, Bagwell CE, Rozycki HJ (1999) Circulating Pro- and Counterinflammatory Cytokine Levels and Severity in Necrotizing Enterocolitis. Pediatrics 103:766–771.

EFSA (2012) Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. EFSA Journal 10:2740.

Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, Urbanek R, Szépfalusi Z (2010) Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr Allergy Immunol 21:1179–88.

Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M, Gil A, Vieites JM, Norin E, Young D, Scott J a, Doré J, Edwards C (2011) Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology 157:1385–92.

Falony G, Vlachou A, Verbrugghe K, De Vuyst L (2006) Cross-feeding between *Bifidobacterium longum* BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose. Appl Environ Microbiol 72:7835–41.

Fan W, Huo G, Li X, Yang L, Duan C, Wang T, Chen J (2013) Diversity of the intestinal microbiota in different patterns of feeding infants by Illumina high-throughput sequencing. World J Microbiol Biotechnol 29:2365–72.

Fanaro S, Chierici R, Guerrini P, Vigi V (2003) Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl 91:48–55.

Field CJ (2005) The Immunological Components of Human Milk and Their Effect on Immune Development in Infants. J Nutr 135:1–4.

Food and Agriculture Organization of the United Nations and World Health Organization. Report of a Joint FAO/WHO Working group on drafting guidelines for the evaluation of probiotics in food, London, Ontario, Canada. 2002. Available at: ftp://ftp.fao.org/es/esn/food/wgreport2(accessed on April 15, 2012).

Forssten SD, Sindelar CW, Ouwehand AC (2011) Probiotics from an industrial perspective. Anaerobe 17:410–3.

Fouhy F, Guinane CM, Hussey S, Wall R, Ryan CA, Dempsey EM, Murphy B, Ross RP, Fitzgerald GF, Stanton C, Cotter PD (2012) High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob Agents Chemother 56:5811–20.

Gavini F, Pourcher A-M, Neut C, Monget D, Romond C, Oger C, Izard D (1991) Phenotypic Differentiation of Bifidobacteria of Human and Animal Origins. Int J Syst Bacteriol 41:548–557.

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–12.

González-Rodríguez I, Ruiz L, Gueimonde M, Margolles A, Sánchez B (2013) Factors involved in the colonization and survival of bifidobacteria in the gastrointestinal tract. FEMS Microbiol Lett 340:1–10.

Grandy G, Medina M, Soria R, Terán CG, Araya M (2010) Probiotics in the treatment of acute rotavirus diarrhoea. A randomized, double-blind, controlled trial using two different probiotic preparations in Bolivian children. BMC Infect Dis 10:253.

Guarner F, Schaafsma GJ (1998) Probiotics. Int J Food Microbiol 39:237–8.

Guarner F (2006) Enteric Flora in Health and Disease. Digestion 73: 5-12.

Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilán CG, Margolles A (2009) Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. Appl Environ Microbiol 75:3153–60.

Haarman M, Knol J (2005) Quantitative Real-Time PCR Assays To Identify and Quantify Fecal *Bifidobacterium* Species in Infants Receiving a Prebiotic Infant Formula Quantitative Real-Time PCR Assays To Identify and Quantify Fecal *Bifidobacterium* Species in Infants Receiving a Prebio.

Hansen CHF, Nielsen DS, Kverka M, Zakostelska Z, Klimesova K, Hudcovic T, Tlaskalova-Hogenova H, Hansen AK (2012) Patterns of early gut colonization shape future immune responses of the host. PLoS One 7:e34043.

Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ (2004) Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut 53:1602–9.

Hauer J, Anderer FA (1993) Mechanism of stimulation of human natural killer cytotoxicity by arabinogalactan from Larix occidentalis. Cancer Immunol Immunother 36:237–44.

HC (2006). Health Canada. Evidence for safety and efficacy of finished natural health products. Ottawa (ON): Natural Health Products Directorate, Health Canada. [Accessed 2008 May 28]. (Available from: http://www.hc-sc.gc.ca/dhp-mps/prodnatur/legislation/docs/efe-paie_e.html).

He F, Ouwehand AC, Isolauri E, Hashimoto H, Benno Y, Salminen S (2001) Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. FEMS Immunol Med Microbiol. 30:43–7.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014) Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514.

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr 73:365S–373.

Hooper L V (2004) Bacterial contributions to mammalian gut development. Trends Microbiol 12:129–34.

Hooper L V, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. Science 336:1268–73.

Hosono A, Ozawa A, Kato R, Ohnishi Y, Nakanishi Y, Kimura T, Nakamura R (2003) Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. Biosci Biotechnol Biochem 67:758–64.

Hunter CJ, Upperman JS, Ford HR, Camerini V (2008) Understanding the susceptibility of the premature infant to necrotizing enterocolitis (NEC). Pediatr Res 63:117–23.

Jankowska A, Laubitz D, Antushevich H, Zabielski R, Grzesiuk E (2008) Competition of *Lactobacillus paracasei* with Salmonella enterica for adhesion to Caco-2 cells. J Biomed Biotechnol 2008:357964.

Jost T, Lacroix C, Braegger CP, Chassard C (2012) New insights in gut microbiota establishment in healthy breast fed neonates. PLoS One 7:e44595.

Kagnoff MF (2007) Review series Celiac disease: pathogenesis of a model immunogenetic disease.

Kelly D, Conway S, Aminov R (2005) Commensal gut bacteria: mechanisms of immune modulation. Trends Immunol 26:326–33.

Klaassens ES, Boesten RJ, Haarman M, Knol J, Schuren FH, Vaughan EE, de Vos WM (2009) Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants. Appl Environ Microbiol 75:2668–76.

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE (2011) Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 108 Suppl :4578–85.

Koletzko S, Osterrieder S (2009) Acute infectious diarrhea in children. Dtsch Arztebl Int 106:539–47.

Kolida S, Tuohy K, Gibson GR (2002) Prebiotic effects of inulin and oligofructose. Br J Nutr 87 Suppl 2:S193–7.

Laparra JM, Sanz Y (2010) Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacol Res 61:219–25.

Le Roy T, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, Martin P, Philippe C, Walker F, Bado A, Perlemuter G, Cassard-Doulcier A-M, Gérard P (2013) Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. Gut 62:1787–94.

Lee JS, Polin R (2003) Treatment and prevention of necrotizing enterocolitis. Semin Neonatol 8:449–59.

Lee YK, Margolles A, Mayo B et al. (2008) Probiotic microorganisms. In: Handbook of Probiotics and Prebiotics, Lee YK and Salminen S (eds.) John Wiley & Sons, NJ, pp. 1–176.

Lievin V (2000) *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47:646–652.

Lilly DM, Stillwell RH (1965) Probiotics: Growth-Promoting Factors Produced by Microorganisms. Science (80-) 147:747–748.

Lin H-C, Hsu C-H, Chen H-L, Chung M-Y, Hsu J-F, Lien R, Tsao L-Y, Chen C-H, Su B-H (2008) Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. Pediatrics 122:693–700.

Lowenthal A, Livni G, Amir J, Samra Z, Ashkenazi S (2006) Secondary bacteremia after rotavirus gastroenteritis in infancy. Pediatrics 117:224–6.

Macfarlane S, Macfarlane GT, Cummings JH (2006) Review article: prebiotics in the gastrointestinal tract. Aliment Pharmacol Ther 24:701–14.

Magne F, Hachelaf W, Suau A, Boudraa G, Bouziane-Nedjadi K, Rigottier-Gois L, Touhami M, Desjeux J-F, Pochart P (2008) Effects on faecal microbiota of dietary and acidic oligosaccharides in children during partial formula feeding. J Pediatr Gastroenterol Nutr 46:580–8.

Mai V, Young CM, Ukhanova M, Wang X, Sun Y, Casella G, Theriaque D, Li N, Sharma R, Hudak M, Neu J (2011) Fecal microbiota in premature infants prior to necrotizing enterocolitis. PLoS One 6:e20647.

Mai V, Torrazza RM, Ukhanova M, Wang X, Sun Y, Li N, Shuster J, Sharma R, Hudak ML, Neu J (2013) Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. PLoS One 8:e52876.

Mangin I, Suau A, Gotteland M, Brunser O, Pochart P (2010) Amoxicillin treatment modifies the composition of *Bifidobacterium* species in infant intestinal microbiota. Anaerobe 16:433–8.

Manning TS, Gibson GR (2004) Microbial-gut interactions in health and disease. Prebiotics. Best Pract Res Clin Gastroenterol 18:287–98.

Marcobal A, Barboza M, Froehlich JW, Block DE, German JB, Lebrilla CB, Mills DA (2010) Consumption of human milk oligosaccharides by gut-related microbes. J Agric Food Chem 58:5334–40.

Mason KL, Huffnagle GB, Noverr MC, Kao JY (2008) Overview of gut immunology. Adv Exp Med Biol 635:1–14.

Medzhitov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol 1:135–45.

Metchnikoff E (1908) The prolongation of life. Putnam, New York.

Mitsuoka T (1984) Taxonomy and Ecology of Bifidobacteria. Bifidobact Microflora 3:11–28.

Moles L, Gómez M, Heilig H, Bustos G, Fuentes S, de Vos W, Fernández L, Rodríguez JM, Jiménez E (2013) Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. PLoS One 8:e66986.

Montalto M, D'Onofrio F, Gallo A, Cazzato A, Gasbarrini G (2009) Intestinal microbiota and its functions. Dig Liver Dis Suppl 3:30–34.

Mshvildadze M, Neu J, Shuster J, Theriaque D, Li N, Mai V (2010) Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. J Pediatr 156:20–5.

Neutra MR, Mantis NJ, Kraehenbuhl JP (2001) Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol 2:1004–9.

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7:688–93.

O'Toole PW, Claesson MJ (2010) Gut microbiota: Changes throughout the lifespan from infancy to elderly. Int Dairy J 20:281–291.

Ouwehand A, Vesterlund S (2003) Health aspects of probiotics. IDrugs 6:573–80.

Parisi G, Bottona E, Carrara M, Cardin F, Faedo A, Goldin D, Marino M, Pantalena M, Tafner G, Verdianelli G, Zilli M, Leandro G (2005) Treatment Effects of Partially Hydrolyzed Guar Gum on Symptoms and Quality of Life of Patients with Irritable Bowel Syndrome. A Multicenter Randomized Open Trial. Dig Dis Sci 50:1107–1112.

Patterson J, Burkholder K (2003) Application of prebiotics and probiotics in poultry production. Poult Sci 82:627–631.

Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I, van den Brandt P a, Stobberingh EE (2006) Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118:511–21.

Penttila IA (2010) Milk-derived transforming growth factor-beta and the infant immune response. J Pediatr 156:S21–5.

Perdigón G, Maldonado Galdeano C, Valdez JC, Medici M (2002) Interaction of lactic acid bacteria with the gut immune system. Eur J Clin Nutr 56 Suppl 4:S21–6.

Piddock LJ V (2006) Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol 4:629–36.

Pierre F, Perrin P, Champ M, Bornet F, Meflah K, Menanteau J (1997) Short-Chain Fructo-oligosaccharides Reduce the Occurrence of Colon Tumors and Develop Gut-associated Lymphoid Tissue in Min Mice. Cancer Res 57:225–228.

Rastall RA, Gibson GR, Tannock GW (2002) Prebiotic oligosaccharides: evaluation of biological activities and potential future developments. 107–148.

Rijkers GT, Bengmark S, Enck P, Haller D, Herz U, Kalliomaki M, Kudo S, Lenoir-Wijnkoop I, Mercenier A, Myllyluoma E, Rabot S, Rafter J, Szajewska H, Watzl B, Wells J, Wolvers D, Antoine J-M (2010) Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. J Nutr 140:671–676.

Roberfroid M (2007) Prebiotics: The Concept Revisited. J Nutr 137:830-837.

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco M-J, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A (2010) Prebiotic effects: metabolic and health benefits. Br J Nutr 104 Suppl :S1–63.

Roberfroid M, Slavin J (2010) Nondigestible Oligosaccharides. Crit. Rev. Food Sci. Nutr.

Robinson RR, Feirtag J, Slavin JL (2001) Effects of dietary arabinogalactan on gastrointestinal and blood parameters in healthy human subjects. J Am Coll Nutr 20:279–85.

Romano C, Comito D, Famiani A, Calamarà S, Loddo I (2013) Partially hydrolyzed guar gum in pediatric functional abdominal pain. World J Gastroenterol 19:235–40.

Ruas-Madiedo P, Hernández-Barranco A, Margolles A, de los Reyes-Gavilán CG (2005) A bile salt-resistant derivative of *Bifidobacterium animalis* has an altered fermentation pattern when grown on glucose and maltose. Appl Environ Microbiol 71:6564–70.

Ruiz L, Margolles A, Sánchez B (2013) Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Front Microbiol 4:396.

Rycroft CE, Jones MR, Gibson GR, Rastall RA (2001) A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. J Appl Microbiol 91:878–887.

Saad N, Delattre C, Urdaci M, Schmitter JM, Bressollier P (2013) An overview of the last advances in probiotic and prebiotic field. LWT - Food Sci Technol 50:1–16.

Saavedra J., Bauman N., Perman J., Yolken R., Oung I (1994) Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 344:1046–1049.

Saavedra JM (2007) Use of Probiotics in Pediatrics: Rationale, Mechanisms of Action, and Practical Aspects. Nutr Clin Pract 22:351–365.

Samadi R (1998) Replacement of intravenous therapy by oral rehydration solution in a large treatment centre for diarrhoea with dehydration. Bull World Health Organ 76:319.

Sánchez B, Ruiz L, Gueimonde M, Ruas-Madiedo P, Margolles A (2013) Adaptation of bifidobacteria to the gastrointestinal tract and functional consequences. Pharmacol Res 69:127–36.

Saulnier DMA, Spinler JK, Gibson GR, Versalovic J (2009) Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Curr Opin Biotechnol 20:135–41.

Savino F, Cresi F, Pautasso S, Palumeri E, Tullio V, Roana J, Silvestro L, Oggero R (2004) Intestinal microflora in breastfed colicky and non-colicky infants. 825–829.

Savino F, Pelle E, Palumeri E, Oggero R, Miniero R (2007) *Lactobacillus reuteri* (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. Pediatr 119:124–130.

Savino F, Cordisco L, Tarasco V, Calabrese R, Palumeri E, Matteuzzi D (2009) Molecular identification of coliform bacteria from colicky breastfed infants. Acta Paediatr 98:1582–8.

Savino F, Cordisco L, Tarasco V, Palumeri E, Calabrese R, Oggero R, Roos S, Matteuzzi D (2010) *Lactobacillus reuteri* DSM 17938 in infantile colic: a randomized, double-blind, placebo-controlled trial. Pediatrics 126:e526–33.

Savino F, Tarasco V (2010) New treatments for infant colic. Curr Opin Pediatr 22:791–7.

Savino F, Cordisco L, Tarasco V, Locatelli E, Di Gioia D, Oggero R, Matteuzzi D (2011) Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC microbial 11:157.

Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen M-C, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 99:14422–7.

Schnabl K-L, Van Aerde J-E, Thomson A-B, Clandinin M-T (2008) Necrotizing enterocolitis: a multifactorial disease with no cure. World J Gastroenterol 14:2142–61.

Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, Price NP, Richardson PM, Mills DA (2008) The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A 105:18964–9.

Sela DA, Mills DA (2010) Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. Trends Microbiol 18:298–307.

Sherman MP (2010) New concepts of microbial translocation in the neonatal intestine: mechanisms and prevention. Clin Perinatol 37:565–79.

Soccol CR, Vandenberghe LP de S, Spier MR, Medeiros ABP, Yamaguishi CT, Lindner JDD, Pandey A, Thomaz-Soccol V (2010) The Potential of Probiotics: A Review. Food Technol Biotechnol 48:413–434.

Songjinda P, Nakayama J, Kuroki Y, Tanaka S, Fukuda S, Kiyohara C, Yamamoto T, Izuchi K, Shirakawa T, Sonomoto K (2005) Molecular monitoring of the developmental bacterial community in the gastrointestinal tract of Japanese infants. Biosci Biotechnol Biochem 69:638–41.

Sorokulova IB, Pinchuk I V, Denayrolles M, Osipova IG, Huang JM, Cutting SM, Urdaci MC (2008) The safety of two Bacillus probiotic strains for human use. Dig Dis Sci 53:954–63.

Strauch UG, Obermeier F, Grunwald N, Gürster S, Dunger N, Schultz M, Griese DP, Mähler M, Schölmerich J, Rath HC (2005) Influence of intestinal bacteria on induction of regulatory T cells: lessons from a transfer model of colitis. Gut 54:1546–52.

Sung V, Hiscock H, Tang MLK, Mensah FK, Nation ML, Satzke C, Heine RG, Stock A, Barr RG, Wake M (2014) Treating infant colic with the probiotic Lactobacillus reuteri: double blind, placebo controlled randomised trial. BMJ 348:g2107.

Szajewska H, Ruszczyński M, Radzikowski A (2006) Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. J Pediatr 149:367–372.

Takeda K, Akira S (2005) Toll-like receptors in innate immunity. Int Immunol 17:1–14.

Tanoue T, Nishitani Y, Kanazawa K, Hashimoto T, Mizuno M (2008) In vitro model to estimate gut inflammation using co-cultured Caco-2 and RAW264.7 cells. Biochem Biophys Res Commun 374:565–9.

Tao N, Wu S, Kim J, An HJ, Hinde K, Power ML, Gagneux P, German JB, Lebrilla CB (2011) Evolutionary glycomics: characterization of milk oligosaccharides in primates. J Proteome Res 10:1548–57.

Tham CS-C, Peh K-K, Bhat R, Liong M-T (2011) Probiotic properties of bifidobacteria and lactobacilli isolated from local dairy products. Ann Microbiol 62:1079–1087.

Tissier MH (1899) La réaction chromophile d'Escherich et Bacterium Coli C. R. Soc Biol 51: 943–945.

Tuohy KM, Kolida S, Lustenberger AM, Gibson GR (2007) The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructo-oligosaccharides – a human volunteer study. Br J Nutr 86:341.

Turroni F, Bottacini F, Foroni E, Mulder I, Kim J-H, Zomer A, Sánchez B, Bidossi A, Ferrarini A, Giubellini V, Delledonne M, Henrissat B, Coutinho P, Oggioni M, Fitzgerald GF, Mills D, Margolles A, Kelly D, van Sinderen D, Ventura M (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 107:19514–9.

Turroni F, Peano C, Pass D a, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M (2012a) Diversity of bifidobacteria within the infant gut microbiota. PLoS One 7:e36957.

Turroni F, Strati F, Foroni E, Serafini F, Duranti S, van Sinderen D, Ventura M (2012b) Analysis of predicted carbohydrate transport systems encoded by *Bifidobacterium bifidum* PRL2010. Appl Environ Microbiol 78:5002–12.

Vandenplas Y, De Hert SG (2011) Randomised clinical trial: the synbiotic food supplement Probiotical vs. placebo for acute gastroenteritis in children. Aliment Pharmacol Ther 34:862–7.

Walker A (2010) Breast milk as the gold standard for protective nutrients. J Pediatr 156:S3–7.

Wallace B (2009) Clinical use of probiotics in the pediatric population. Nutr Clin Pract 24:50–9.

Westerbeek EAM, van den Berg A, Lafeber HN, Knol J, Fetter WPF, van Elburg RM (2006) The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 25:361–8.

Whisner CM, Martin BR, Schoterman MHC, Nakatsu CH, McCabe LD, McCabe GP, Wastney ME, van den Heuvel EGHM, Weaver CM (2013) Galacto-oligosaccharides increase calcium absorption and gut bifidobacteria in young girls: a double-blind cross-over trial. Br J Nutr 110:1292–303.

WHO (2001) Fifty-fourth World Health Assembly. WHA54.2. Agenda item 13.1. Infant and young child nutrition.

Wiegering V, Kaiser J, Tappe D, Weissbrich B, Morbach H, Girschick HJ (2011) Gastroenteritis in childhood: a retrospective study of 650 hospitalized pediatric patients. Int J Infect Dis 15:e401–7.

Wostmann B S (1996) Germfree and Gnotobiotic Animal Models: Background and Applications. Boca Raton, FL: CRC Press. pp 101-125.

Yang H, Yu Y, Song F, Liu S (2011) Structural characterization of neutral oligosaccharides by laser-enhanced in-source decay of MALDI-FTICR MS. J Am Soc Mass Spectrom 22:845–55.

Yun JW (1996) Fructooligosaccharides-Occurrence, preparation, and application. Enzyme Microb Technol 19:107–117.