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Therapeutic microbiology: characterization of *Bifidobacterium* strains for the treatment of enteric disorders in newborns

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PART 1: INTRODUCTION

Chapter 1. Intestinal microbiota in early infancy: composition and development

1.1 Microbiota composition in early infancy

The intestinal microbiota of humans is a specific ecosystem made of a complex array of microorganisms (~ 10^{14} – 10^{15} CFU/g of lumen content) which forms an individual microbiota typical for each being . In particular, the human intestinal microbiota consists of more than 400 different species.

Birth brings about an immediate end to the sterility of the fetus enviroment: microbial colonization begins after birth, within a few hours bacteria start to appear in the feces. Studies of gnotobiotic mice have been particularly enlightening, illustrating the essential role of the gastrointestinal microbiota in normal gut development (Ley et al., 2006).Thaks to these studies, it is argued that the microbial diversity of the human gut is the result of coevolution between microbial communities and their hosts and that the peculiar structure of microbial diversity in the human gut resulted from natural selection operating at two different levels: the host level selection on the community which favours stable societies with a high degree of functional redundancy and a selection pressure driving microbial cells to become functionally specialized.

The first microbial population the newborn comes into contact with are the maternal intestinal and vaginal microbiota; successively, the newborn will be exposed to the microbes from the environment. Still, the microbial colonization of the infant gastrointestinal tract (GIT) is a remarkable episode in the human lifecycle.

A low amounts of bacteria is encountered a few hours after birth; the main bacteria genera isolated at these time are *Staphylococcus*, *Streptococcus*, *Propionibacterium*, *Corynebacterium*. Following a rupture of the fecal membranes, bacteria of maternal origin can be isolated.

The first bacteria encountered in the majority of healthy infants are facultative anaerobes, because the intestinal environmental of neonates shows a positive oxidation/reduction potential at birth. These bacteria remain predominant during the first few days of life, among them, *Staphylococcus*, *Enterobacteriaceae* and *Streptococcus* are the genera most commonly isolated from the newborn faeces at birth.

Gradually the consumption of oxygen by these bacteria changes the intestinal environment into a more-reduced one, permitting the subsequent growth of strict anaerobes (Bezirtzoglou, 1997). Facultative anaerobic bacteria are followed by *Bifidobacterium* spp., *Bacteroides* spp. and *Clostridium* spp. which are present within 2 days with an increased incidence in newborns delivered by a Caesarean section. In fact, in comparison with vaginal delivery, cesarean section resulted in lower colonization rates and counts of bifidobacteria and *Bacteroides fragilis* group species, whereas counts of *Clostridium difficile* and *Escherichia coli* are higher. The presence of *C. difficile* is impoetant for the installation of other anaerobic putrefactive microorganisms such as other bacteria belonging to the *Clostridium* genus.

As already mentioned before, the microbial population of the newborn changes in relation to many factors like diet (breast versus formula feeding), mode of delivery (natural delivery versus caesarean delivery), maternal diet, antibiotic use during the first few months of life and early enviromental surroundings (**Table 1**).

In recent year a first large epidemiologic study (KOALA study) on determinants of gut microbial composition in early infancy was carried out in the Netherlands (Penders et al., 2006). Within the KOALA project fecal samples of 1000 infants, 1 month of age, were analyzed in order to study the potential determinants in a multivariate manner and to distinguish their independent effects. Participants at 34 weeks of gestation with diverse lifestyles, i.e. pregnant women with a conventional lifestyle and pregnant women with an alternative lifestyle women that consume only organic food, follow Steiner principles and alternative medicines, were recruited.

In agreement with previous researches, the KOALA study confirm that term infants who were born vaginally at home and were exclusively breastfed seemed to have the most “beneficial” gut microbiota, with the highest numbers of bifidobacteria and lowest numbers of *C. difficile* and *E. coli.* Conversely, lifestyle appears not to influence gut microbial composition.

Table 1 Principal factors influencing intestinal microbiota development in newborns

Factors
Place and mode of delivery
Maternal microbiota of intestine, vagina and epidermis
Type of infant feeding
Antibiotic/antimycotic use
Gestational age at birth
Hospitalization after birth

1.2 Influence of the mode of delivery on the infant microbiota composition

The environment is extremely important for intestinal colonization of infants born by cesarean section. Cesarean section new borns do not come in contact with the maternal vaginal and faecal microrganisms and may be separated from the mother for a long period after birth (Biasucci et al., 2010). In this situation the environment becames a crucial source of colonizing bacteria. These bacteria are mainly introduced from the environment of the hospital although it is known that bacteria introduced from the hospital environment have a low colonization ability during the first 7 days of life.

Anaerobic colonization, especially by *Bacteroides* spp. is delayed but *Bifidobacterium* retrieval and *E.coli* presence was similar in vaginally and caesarean section delivered infants. Additionally, an increased incidence of *Clostridium perfringens* and *C. difficile* is reported in relation to the hospital environment (Penders et al., 2006).

Environmental contamination seems to be the main route for clostridial implantation in the newborn and the rapid implantation of *C. perfringens* in cesarean sectioned newborns seems to determine a decrease in redox potential which favors the subsequent colonization by anaerobic bacteria like other species of *Clostridium* and *Bacteroides* spp..

1.3 Effects of infant feeding on the gut microbiotic composition in infants

Another important factor that can influence composition of the intestinal microbiota in the neonates is the type of feeding. Also in the KOALA study it has been demonstrated that diet can have an influence on the gut microbiota.

In both breast- and formula-fed infants, the GIT is initially colonized by streptococci and enterobacteria and these create anaerobic condition necessary for the establishment of the anaerobic *Bacteroides* spp. and *Bifidobacterium* spp. In full term breastfed neonates *Bacteroides* spp., bifidobacteria can appear 4 days after birth and after 1 week they dominate the faecal microbiota of breast-fed infants and their counts increase rapidly to constitute 80%-90% of the total flora. In contrast, the faecal microbiota of the 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, a number of peptides capable of stimulating the growth of several bifidobacteria have recently been isolated from human milk. Another factor that could contribute to the dominance of bifidobacteriain the faeces of breast-fed infants is the presence in the human milk of glucoprotein, glycolipids, fucose, neuraminic acid, lactose, N-acetylglucosamine, and, a variety of oligosaccharides (Coppa and Gabrielli, 2008).

Both adults and neonates are regularly exposed to microorganisms via the diet, but with different effects. The microorganisms entering newborns throught breast milk are more likely to colonize than those entering in healthy adults with stable climax communities are. However, the results available to date on bifidogenic effects of milk molecules are still inconclusive and there is also a lack of information about the isolation and identification of commensal or potential probiotics bacteria, including bifidobacteria, from milk of healthy women. Even though authors are aware that human milk is difficult to sample and microbial contamination can never be totally discarded, some

studies have demostrated the presence of alive bifidobacteria in human milk (Martin et al., 2003, Solis et al., 2010).
It has been hypothesized, within the KOALA study, that the maternal diet not only might be a determinant of the mother's gut microbiota but also might influence her infant's gut microbiotic composition. However, no association between maternal use of probiotics during pregnancy and the intestinal microbiotic composition at the age of 1 month was found (Penders et al., 2006).
Recent studies have been demonstrated that another additional anaerobic bacterial group is to be considered as dominant in breast-fed babies during the first days of life, i.e. *Ruminococcus* (Morelli, 2008). It is also interesting to note that ruminococci seem to be positively affected by oligosaccharides, at least in animal models. The complete role of ruminococci in protecting the health of babies is far from being understood, anyway *Ruminococcus* is recognized to have an important protective effect on the host because it produces ruminococcin A, a bacteriocin that can inhibit the development of many species of *Clostridium*.

1.4 The intestinal bacterial colonization in preterm infants

In contrast with full term neonates, little information concerning the composition of the microbiota in premature infants is available because only a few studies have determined the developmental aspects of the intestinal colonization in these subjects. It is difficult to draw firm conclusion on the fecal microbial community in preterm infants for several reasons: the inter-individual variability is very high and many parameters, such as antibiotic regiments and diet, may tend to increase study discrepancy. In particular, preterm 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.
In addition, the limited number of patients analyzed usually do not allow to fully understand the microbiota composition. As this category of infants often require intensive care treatments due to an increased risk for serious infections, insight in the intestinal colonization is important.
At the first days of life, the preterm infants are predominantly colonized by facultative anaerobic bacteria, which remain at high levels, resembling the full term formula-fed

infants. However, the counts of enterobacteria and enterococci remain predominant until the 20th day of life and significantly higher than in full term breast-fed infants (Magne et al., 2005).

Moreover, 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 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) which affects predominantly premature and low weight newborns (Lin et al., 2008).

1.5 Effects hospitalization on the microbiota composition in infants

Prematurity is strongly associated with hospitalization. In addition, hospitalization itself is incriminated to changing the normal microbiota. Changes in the intestinal microbiota composition upon chemioterapic administration is observed, for example the oral use of antibiotics (mainly amoxicillin) by the infant during the first 1 month of life resulted in decreased numbers of bifidobacteria and *B. fragilis*-group species (Penders et al., 2006; Mangin et al., 2010).

Moreover the simple impact of hospitalization, even without any antibiotic treatment produces changes in the normal microbiota. In hospitalized newborns intestinal colonization by *Klebsiella*, *Proteus*, *Pseudomonas*, as well as *E.coli* occurs more frequently (Penders et al., 2006).

Chapter 2. Interaction between gut microorganisms and intestinal epithelial surface

The microbiota is in close conctact with the intestinal mucosa and epithelia surface which is, after the respiratory area, the largest surface of the body, occupying approximately 250-400 m^2. Some anatomical and physiological aspect of the host organism are directly linked to the presence and activity of the intestinal microbiota such as formation of the intestinal walls, production of organic acids and vitamins, stimulation of immune system etc. The main fuctions of the microbiota on the host organism will be analysed in this chapter.

2.1 Structure and fuctions of intestinal surface

The intestinal mucosal surface is exposed to the lumen and the cells present in the external layer, along with their secretions, form a barrier between non sterile internal environment and the essentially sterility of the body (Duerr and Hornef, 2011) (**Figure 1**). As a result of these exposure the mucosal surfaces are the principal locus of attack by microorganisms.

Mucosa consists of three layers: the first is made up of the epithelial cells, which can be a single layer as in GI tract. The cells are attached to a basement membrane overlying the second layer, the lamina propria, which consists of subepithelial connective tissue and lymph nodes, underneath which is the third layer, a thin layer of smooth muscles called the muscolar mucosa.

The epithelial cells of the GI tract are squamous in the esophageal part but they became leaky and die before being shed into the lumen. This desquamation of the cells is an important mechanism of preventing microrganism invasion. In the intestinal tract the columnar epithelial mucus is secreted by goblet cells interspersed among the enterocytes. Enterocytes are polarized cells with a dinstinct apical and basolateral cytoplasmatic membrane. However the intestinal epithelium also contains M cells, which are present in Peyer's patches and are part of the gut- associate lymphoid tissue. The M cells are specialized epithelial cells that transport antigens and microorganisms from their apical surface throught the cytoplasm to the basolateral surface by using

transcytosis. Immune cells such as macrophages and lymphocytes are located in their extracellular compartement underneath these cells, waiting for antigen presentation.

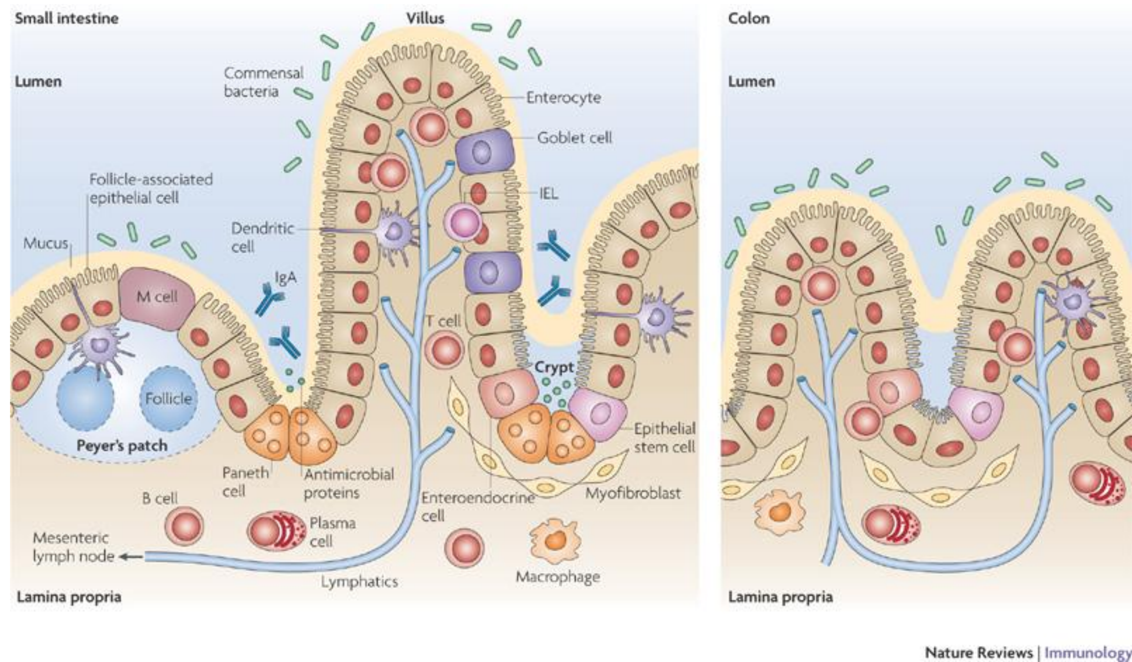


Figure 1 Anatomy of small intestine and colon immune system (Abreu, 2010)

The innate defense system consists of three components: mechanical, chemical, and cellular barriers.

The mechanical barrier is formed by the epithelial cells and the junctions between them (Yu and Yang, 2009). The chemical defence comes from antimicrobial proteins, peptides and cytokines that perform the immune response. The last component of the innate defence is the cellular defence enacted by M cells, dendritic cells, phagocytic cells, mast cells, lymphocytes and epithelial cells (Guarner, 2006).

The first defence that an invading pathogen would encounter is the preepithelial barrier, consisting of a secreted mucus gel. Mucus is therefore a unique physical gel that has both flow and rigidity properties. The secreted mucins are the principals viscous and gel-forming components of the mucus gel secretions. Mucins are high molecular weight glycoproteins.

Using *in vitro* and *in vivo* system (El Asmar et al., 2002; Cencič and Langerholc, 2010) it has been demonstrated that exposure to healthy commensal bacteria results in

establishment of the normal tight-junction barrier between epithelial cells, which represent the major determinant of gut permeability.
In particular the immaturity and the permeability of intestinal epithelial barrier may play a role in pathophysiology of intestinal complications in some neonates and mainly in preterm borns (Stratiki et al., 2007). Among the most severe gastrointestinal complications linked to the weakness of epithelial barrier, there are feeding intolerance, necrotizing enterocolitis (NEC), and gut associated sepsis. These intestinal complications that may occur mostly in the first weeks of life, will be further treated subsequently.

2.2 Protective effects of the gut microbiota on the host

The presence of an abundant commensal microbiota may provide some protection against incoming enteric pathogens and may activate the expression of virulence-related genes (Nataro, 2005).
In addition, experimental data suggest the existence of several complex interacting mechanism in the host defence such as competition with enteric pathogen bacteria for nutrients and adhesions site in the intestinal mucosa and stimulation of the mucosal immune system of the host by activating an appropriate inflammatory response or immune mechanisms against chronic infections (**Figure 2**).

2.2.1 Competition for nutrients between indigenous microbiota and enteric pathogens

The indigenous microbiota gains access to a nutrient enriched, stable environment, and thereby enters a symbiotic relation with the host's intestinal tract. *In vitro* evidence supporting the nutrient-niche hypothesis has been reported by many researcher who used continous flow chemostat culture systems designed to mimic condition of the intestine (Laux et al., 2005). The use of these systems has demonstrated the importance of microbial association in the surfaces, the stability of the population, with respect of major genera, and the role of nutrient utilization in maintaining the population stable. If the analogy of a chemostat is applied to the intestinal tract, several hundred species of bacteria are in equilibrium, competing for resources from an extensive mixture of limiting nutrients, and the only way for a bacterial species to survive is to compete effectively for one or a few of the available nutrients. It's important to remember that

gut is such a rich source of nutrients that it may seem unlike that this is the way in which the gut microbiota influences its own composition. However, it requires only one nutrient to be limiting for this mechanism to operate successfully. *In vitro* results suggest that probiotic microorganisms compete more efficiently than *C. difficile* for monomeric glucose, N-acetyl-glucosamine, and sialic acid found in the colonic contents (Fuller, 1991).

Furthermore, some polysaccharides which can occur naturally (e.g. in breast milk) or are used as food additives can enter in the colon indigested and they are able to stimulate the proliferation only of certain commensal bacteria like lactobacilli and bifidobacteria (Forchielli and Walker, 2005), this topic will be further treated below (see chapter 7).

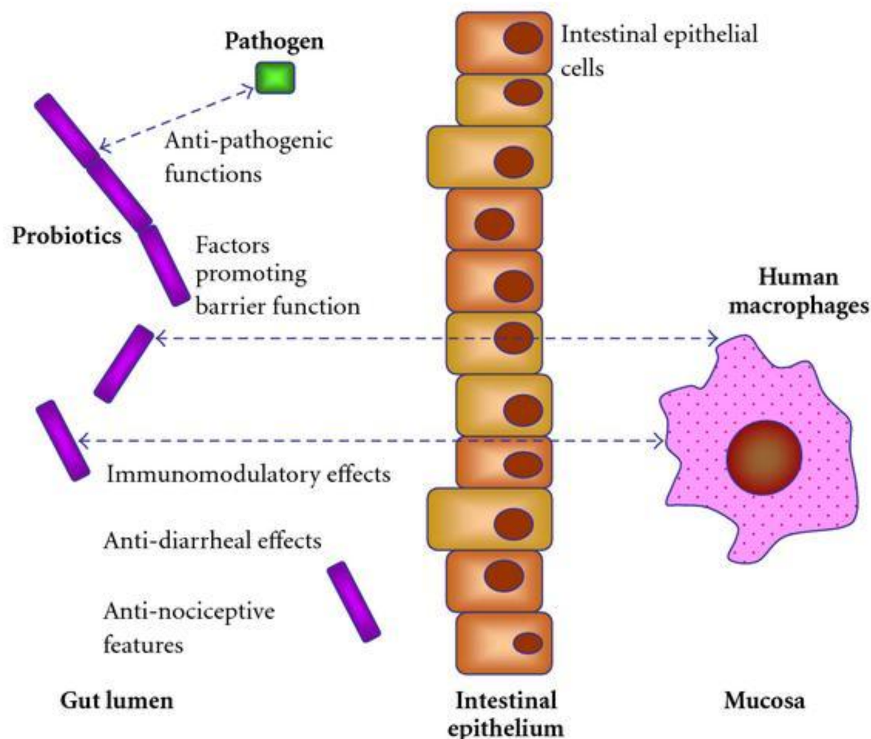


Figure 2 Host defence against intestinal pathogenic bacteria (Britton and Versalovic, 2008).

2.2.2 Competition for intestinal adhesion sites

Adhesion to and colonization of the mucosal surfaces are possibly protective mechanisms against pathogens throught the competition of the binding sites. The ability of some potential probiotic strains belonging to the *Bifidobacterium* and *Lactobacillus* genera to strongly adhere to the intestinal mucosa has been widely studied in the last years (Collado et al., 2005, Del Re et al., 2000, Jankowska et al., 2008) . In particular, bacteria, that are able to adhere to mucus and unable to reach the epithelial cells, might be dislodged from the mucosal surface and washed away with the luminal contents. Indeed there are species of the normal human gut, often introduced in diary products like commercial strains, which should be carefully selected and characterized also for the adhesion to the mucosal surfaces. Many studies used enterocyte-like Caco-2 and HT29 cell lines to investigate the adherence of a large number of *Lactobacillus* and *Bifidobacterium* strains (Del Re et al., 2000, Gopal et al., 2001, Candela et al., 2008,Cencič and Langerholc, 2010).

However, a wide bibliography shows that the displacement activity exerted by probiotic bacteria towards enteropathogens is related to mechanisms other than mere competition for common adhesion sites. Lievin et al. (2000) have demonstrated that *Bifidobacterium* strains isolated from infants produce antibacterial lipophilic factor(s) effective in inhibiting *S. enterica* serovar Typhimurium invasion of Caco-2 cells and in killing intracellular enteropathogenic cells. Fujiwara et al. (2001) have purified a proteinaceous factor that inhibits *in vitro* adherence of an enterotoxigenic E. *coli* strain to gangliotetraosylceramide molecules, which are physiological constituents of the mammalian intestinal epithelium surface.

2.2.3 Stimulation of mucosal immune system

The communication between intestinal microorganisms and the GI epithelium has been extensively studied in the last decades using *in vitro* models and germfree animals. These studies showed that in the absence of the microorganisms, the intestinal immune system is underdeveloped and the morphology is disrupted (Wostmann, 1996), furthermore the germfree animals presented hypoplastic peyer's patches and, a great reduction of immunoglobulin-A producing plasma cells (Macpherson and Harris, 2004) . They also exhibit an altered gene-expression profile of the intestinal epithelial cells.

Results of additional studies suggested that the indigenous intestinal microbiota in mammalians might contribute to the development of both humoral and cellular mucosal immune systems (Hooper, 2004). These interactions maintain a physiologically controlled inflammation or activation of gut-associated lymphoid tissue thoughout life (Neish et al., 2000).

The immune system is able to detect microorganisms by discriminating between self and nonself organisms. This discrimination is possible throught a sophisticated system of receptors that are called Toll-like receptors (TLRs), which provide considerable specificity for pathogen microorganisms. As soon as TLRs provide the alarm signal of infection, the host reacts with an immediate immune response system (Vinderola et al., 2005).TLRs are expressed by macrophages, dendritic cells, endothelial and epithelial cells and they are specialized in different classes like TLR4 that recognizes lipopolisaccarides (LPS) and gram-negative bacteria and TLR2 that recognizes a variety of microbial components such as peptidoglycan and lipoteichoic acids from gram-positive bacteria (Takeda and Akira, 2005).

Furthermore *in vitro* and *in vivo* finding allowed to analyze the secretion of interleukine-6 (IL-6) in responce to bacterial infection (Miller et al., 2002). IL-6 is a multifunctional cytokine involved in diverse biological processes, such as host response to enteric pathogens, acute-phase reaction , hematopoiesis, growth factor for normal or neoplasic cells, and terminal differentiation of B lymphocytes: IL-6 is condidered the product of proinflammatory cells (Montier et al., 2012). By now it is well known that the interaction between probiotics and intestinal cells could play an important role in the innate immune response induced by probiotics (Vinderola et al., 2005, Cencič and Langerholc, 2010).

Much has been learned during recent years about the capability of probiotic strains to induce IL_6 production from epithelial cells (Nissen et al., 2009) and it has been also demonstrated that LAB and bifidobacteria are able to use TLRs to send immune signals to the cells. It was reported that intestinal epithelial cells may be an important source of IL-1ß, IL-6 and IL-8 and that adherent population of Peyer's patches was responsible for the production of gamma interferon (INF-γ) and tumor necrosis factor alpha (TNF-α) (Perdigon et al., 2002, Tanoue et al., 2008) (**Figure 3**).

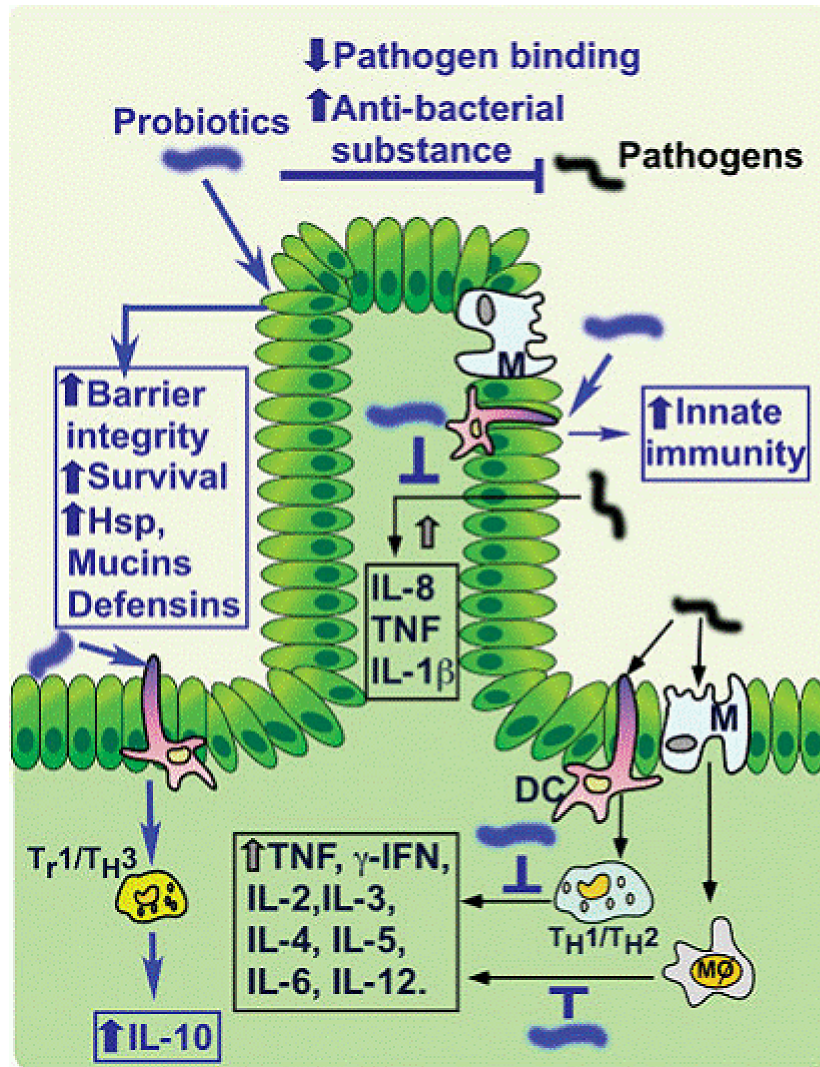


Figure 3 Innate and cell-mediated immune response (Vanderpool et al., 2008).

In addition, reactive oxygen species (ROS) are classically thought of as cytotoxic and mutagenic molecules or as inducers of oxidative stress; recent evidence suggests that ROS play a role in signal transduction. ROS are implicated in stimulation or inhibition of cell proliferation, apoptosis, and cell senescence, moreover they can play an important role in host defence againts infections. Of particular interest, the production of NO and H_2O_2 by epithelial cells and macrophages mediates killing or growth inhibition of bacteria, fungi and parasites (Park et al., 1999; Pipenbaher et al., 2009). The ROS compounds take part in the innate immune response (Keyaerts et al., 2004) and recent studies showed that some probiotic strains increase the production of ROS in small intestinal epithelial cells and in macrophages (Nissen et al., 2009, Pipenbaher et al., 2009, Maragkoudakis et al., 2010).

Moderate production of H_2O_2 and NO induced by probiotics used in food could have a beneficial effect in manteining a balance and increasing resistence to infections. However, it should be noted that hight concentration of H_2O_2 and NO causes tissue injury, disseminated intravascular coagulation and shock (Park et al., 1999).

Lastly, several studies showed that orally administration of lactic acid bacteria (LAB) stimulated IgA secretion and T-cells activation (Perdigon et al., 2001, Dogi et al., 2008), in particular LAB were able to increase IgA cells in a dose dependent manner.

Much of the research on interactions of LAB with epithelial cells has been conducted on tumoral cell lines such as HT-29 and CaCo-2,these studies allowed to better understand some of the complex mechanism of the interaction between microbiota and immune cells.

2.3 Experimental models of gut ecosystem

Human and animal gut is a complex system formed by a large community of microorganisms (intestinal microbiota) that interact with host in the development of intestinal epithelium, in nutrient acquisition and metabolism and in the development of host immune system; because of this complexity it is difficult to find an appropriate experimental model. Germ free animal models have been widely used till recent years but even if they are a good realistic model for such studies they presents major disadvantages like the disagreement with the bioethical spirit of reduce animal testing of EU. Their use is also not suitable in all laboratories because special facilities and special trained personnel are needed. They are also very expensive and ultimely it is not always possible to find a good human model for some of these kind of studies like, for example, pathogen studies (Cencič and Langerholc, 2010) .

As fully described by Cencič and Langerholc, (2010), *in vitro* cell models of the gut should functionally resemble the *in vivo* situation. Primary cells isolated from human or animal tissue conserve the majority of the *in vivo* ecosystem functionality, however the primary cells usually survive only a few days in *in vitro* culture. Primary cells derived from different individuals keep the diversity that is reflected on the results.

Anyway, *in vitro* cell models satisfy basic requirements: availability and easy handling and good human predictive power (Cencič and Langerholc, 2010). Moreover cell

models formed by a combination of epithelial and other cell lines responde to envimental factors like cytokines and inflammatory molecules.

As stated above, in most of the *in vitro* studies of the gut , human colon tumorigenic cell lines like Caco2, T84 and HT-29 have been widely used for mechanistic and functional studies of the gut. However, it is well assessed that the phenotype of tumorigenic cell lines traditionally used for this purpose distinguishes them profoundly from the normal gut epithelium (Tremblay and Slutsky, 2007), in fact adhenocancerogenic cell lines can be altereted in proliferation, glycosilation when compared to non tumorigenic ones. To study the interaction with probiotics and gut epithelium an interesting recent feature is to develop cell culture with non tumorigenic intestinal cells (Cencič and Langerholc, 2010). 3D intestinal epithelial models from various species were developed using both human and animal cell cultures. In particular these 3D models are built from intestinal epithelial cells in a microporous membrane by also adding an underlay of immune cells (macrophage and dendridic cells) that mime the mucosal lymphoid tissue. In the apical side of the membrane, intestinal bacteria can be added in order to makes these models close to *in vivo* situation.

Chapter 3. Principal gastrointestinal diseases in infants and newborns

3.1 Necrotizing enterocolitis (NEC) in infants

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 gastrointestinal dysfunction progressing to pneumatosis intestinalis, systemic shock, and rapid death in severe cases. The most common risk factors cited are prematurity, enteral feeding and bacterial colonization, in particular intestinal injury in NEC may be the results of synergy of these three factors (Claud and Walker, 2001).

However, there is a strong evidence that the initial bacterial colonization after birth plays a pivotal role in the developmentof NEC. As It has been already mentioned before, preterm newborns show a different colonization with respect to full term newborns where *Bifidobacterium* and *Lactobacillus* microrganisms are predominants. In preterm infants more pathogenic microrganisms such as enterobacteria and enterococci remain predominant until the 20th day of life, for this reason one it has been suggested that a major etiological factor for NEC is the abnormal microbiota, particularly as NEC usually occur after 8-9 days postpartum when anaerobic bacteria start to colonize the gut (Mai et al., 2011). It is also true that premature newborns have an immature and inappropriate intestinal ephitelial immunologic response to luminal bacterial stimuli. The observation that immature human enterocytes react with excessive pro-inflammatory cytokine production after inflammatory stimulation can help in part to explain why prematures exposed to initial colonizing bacteria can develop NEC (Nanthakumar et al., 2000).

Several studies have shown that formula–fed infants have a higher incidence of NEC than breast-fed infants, this is due to the fact that breast milk contains passive immunity factors such as polymeric IgA that enhance intestinal maturation and antimicrobial factors providing protection to the newborn.

Moreover the fetal gut is exoposed to amniotic fluid containing hormones and peptides that may have a role in intestinal maturation and preparation for postnatal feeding (Claud and Walker, 2001). The preterm infants may not have this maturation process when initially fed and for that reason they are unable to fully digest carbohydrates and

proteins, leading to the production of organic acids which may be harmful to the developing intestine.
However, despite these scientific evidences the exact etiology and pathogenesis of this disease have not been clearly delineated.

3.2 Bacterial gastroenteritis

Infectious gastroenteritis is one of the leading cause of morbidity especially in newborns and children under 5 years of age. Although gastroenteritis-associated mortality is rare in Western Europe, an increased incidence has been noted in some national registers over recent years (Wiegering et al., 2011). However, acute gastroenteritis vary from place to place depending on local socioeconomic conditions and geography.
Several studies have focused on the etiology of infectious diarrhea in hospitalized newborns and children. Rotavirus is the most common cause of infectious diarrhea in children worldwide, followed by adenovirus and norovirus. The clinical manifestations of viral gastroenteritis include diarrhea, vomiting, fever, anorexia, headache and abdominal cramps. None of these single symptoms clearly distinguishes viral gastroenteritis from diarrheal illness due to bacterial or parasitic organisms.
However, bacterial and viral gastroenteritis present with different clinical features. The differentiation of bacterial *vs.* non-bacterial and rotavirus *vs.* non-rotavirus diarrhea appears to be of particular clinical relevance. Rotavirus infections are known to be more severe and more often associated with a complicated course.
In the last few decades, several enteric bacteria (e.g., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Clostridium difficile*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *E. coli*) and parasites (e.g., *Cryptosporidium spp.*) have been identified as important causes of diarrhea in human, particularly in infants (Amisano et al., 2011).
Diarrheagenic *E.coli* represents one of the most the bacterial cause 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. *E.coli* strains associated with diarrhea have been classified into six groups, based on clinical, epidemiological and molecular criteria: enteropathogenic *E.coli* (EPEC),enteroehaemorragic *E.coli* (EHEC), enteroinvasive

E.coli (EIEC), enterotoxigenic *E.coli* (ETEC), enteroaggregative *E.coli* (EAggEC) and diffusely adherent *E.coli* (DAEC).
A further important etiological agent is *Shigella spp*., that is one of the most common pathogen in children over 1 year of age. Accordingly, *Shigella spp*. (particularly the *S. dysenteriae* and *S. flexneri* serotypes) should also be regarded as a priority target for vaccine development, especially since dysenteric illness is not treated primarily with oral rehydration salts, but usually requires antimicrobial therapy .
K. pneumoniae, E. clocae and *C. difficile*, on the other hands, are normal commensals of the human intestine ubiquitously throughout most of the gut, and they can cause secondary bacteremia notably in the relatively vulnerable intestinal wall of young infants especially after mucosal damage due to rotavirus infection (Lowenthal et al., 2006).
In addition, *Campylobacter* emerged as a significant pathogen, mainly among under-6-month-olds.*Campylobacter* was associated with diarrhoea in some study sites, but mainly among 0-5-month-olds (Allen et al., 2010).

3.3 Infantile colics

Infantile colics are a common condition in the first months of life, about 10-30% of infants are effected by this clinical condition. The classic definition of infantile colic is based on the rule of three: fussy crying that last for > 3 hours per day; for > 3 days per week; and a minimum of 3 weeks. In fact the infant suffers from paroxysms of excessive, highpitched, inconsolable crying, frenquently accompanied by flushing of the face, meteorism, drawing-up of the legs and passing of gas. The crying episodes tend to increase at 6 weeks of age and are most frequent in the evening hours but fortunately this condition usually resolves spontaneously by the age of 3 months. Although infant colic is a common disturbance, the aetiology conditions remain obscure, however evidences suggest multiple independent causes.
The role of an aberrant intestinal microbiota has recently been reproposed to affect gut function and gas production that lead to colicky behaviour. According to Lehtonen et al.,1994, an anomalous microbial composition such as an inadequate bifidobacteria and lactobacilli level in the first months of life may affect the intestinal fatty acid profile thereby favouring the development of infantile colics.

Bifidobacteria and lactobacilli play an important role in the development of local and systemic immune responses in that way an inadequate balance of these microrganisms in colickly infants might underlie immaturity in the gut barrier and lead to aberrant immune responses and increase vulnerability. Furthermore recent studies (Savino et al., 2009) showed that colickly infants have higher counts of anaerobic Gram- negative bacteria than healthy infants and in particular of gas forming coliforms that are rod-shaped organisms that fermente lactose resulting in gas formation at 35-37°C. The most frequent faecal coliform genera are *Escherichia, Enterobacter, Klebsiella* and *Enterococcus.*

Is is feasible that gas coliforms may contribute to colonic fermentation and consequently to excessive intra-intestinal air load, aerophagia and pain, which are the typical symptoms of infant crying, but many aspect of these relationships are still unknown and the contribution of coliforms colonization remains to be clarified (Savino et al., 2007).

Some recent evidences suggest that infantile colics might have many several independent causes, such as lactose intolerance. In this regard, infants, during the first period of life, may display malabsorption of carbohydrates present in breast milk or formula milk and recently, the hypothesis is that colic syntoms could be relieved by reducing the lactose content of the infant feed. According to other new theories, infant colics could be related to food allergy and sometimes could be manifestation of atipic deseases. According to Lindberg, 1999, infants with colic respond favourably to diet free of cow's milk protein. Moreover, a recent trial suggested that a new formula with partially hydrolized proteins, a low amount of lactose and containig a mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) led a significant improvement of infantile gas colics and other gastrointestinal disorders (Savino et al., 2007). As indicated in a dedicated section (4.3), the possibly of reducing the synthoms of colics with the use of probiotics has been explored (Savino et al., 2010).

3.4 Neonatal bacterial infections: group B Streptococcal infection

Early-onset bacterial sepsis remain one of the major cause 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 intrapartum antibiotic prophylaxis in pregnant women during labor and delivery. There are multiple says throught which bacteria can enter and infect newborns: the primary portal appear to be the respiratory tract, however acquisition via placenta is also possible. The leading cause of onset infection of fetus and newborn is group B *Streptococcus* (GBS). This gram-negative bacterium that resides in the cervix, vagine 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.

In 1996, the Centers for Disease Control and Prevention (CDC) published consensus guidelines for the prevention of neonatal GBS disease that approved the use of intrapartum antibiotic prophylaxis (IAP) for a maternal screening (Puopolo et al., 2005). Penicillin is recommended as the first-line agent for intrapartum antibiotic prophylaxis, while ampicillin is considered as an acceptable alternative. In penicillin-allergic women, who are not at high risk for anaphylaxis, clarithromycin and cefazolin are considered the agents of choice for intrapartum chemoprophylaxis because of its narrow spectrum of activity and ability to achieve high intraamniotic concentrations.

In **Table 2** the principal symptoms of the early-onset and late-onset infection have been reported . They are very different: 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, over the past decade with the introduction of antibiotic maternal prophylaxis, there has been a significant decrease in the incidence of GBS to its current rate of approximately 0.32 per 1000 live births for early-onset disease, however there is no evidence that chemoprophylaxis prevents late-onset disease (**Table 2**). However, there are no information in the literature on the effect that the 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.

Table 2 Manifestations of early-onset and late-onset group B streptococcal disease

Characteristic	Early-onset disease	Late-onset disease
Age at onset	Birth to day 6	Day 7 to 3 months
Symptoms	Respiratory distress, apnea	Irritability, fever, poor feeding
Findings	Pneumonia, sepsis	Sepsis, meningitis, osteoarthritis
Mode of trasmission	Vertical, in utero, intrapartum	Nosocomial, horizontal
Effect of antibiotic prophylaxis	Reduce incidence by 85-90%	No effect

Chapter 4. Probiotics

4.1 History of Probiotics

The term *probiotic*, meaning "for life," is derived from the Greek language and it is currently used to name bacteria associated with beneficial effects for humans and animals. The original observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, the Russian Nobel Prize working at the Pasteur Institute at the beginning of the last century; Metchnikoff (1908) in his book "*The Prolongation of Life"* was probably the first one to advocate, or rather postulate, the health benefits of LAB associated with fermented milk products. He hinted that the longevity of the Caucasians could be related to the high intake of fermented milk products and that the intake of yogurt containing lactobacilli might result in a reduction of toxin-producing bacteria in the gut and that this could increase the longevity of the host. Tissier, a French paediatrician, recommended the administration of bifidobacteria to infants suffering from diarrhea, claiming that bifidobacteria supersede the putrefactive bacteria that cause the disease. The expression "probiotic" was probably first defined by Kollath in 1953 (Kollath, 1953), when he proposed the term to identify all organic and inorganic food complexes as "probiotics," in contrast to harmful antibiotics in order to upgrade such food complexes as supplements.

Later, Lilly and Stillwell (1965) identified probiotics as "substances secreted by one microorganism which stimulates the growth of another", against the concept of *antibiotic*. It may be because of this positive and general claim of the definition that the term *probiotic* was subsequently applied to other substances and gained a more general meaning. In 1971 Sperti (Sperti, 1971) applied the term to tissue extracts that stimulate microbial growth. Parker (1974) was the first to use the term *probiotic* in the sense that it is used today. He defined probiotics as "organisms and substances which contribute to intestinal microbial balance." The use of the word *substances* in Parker's definition of probiotics resulted in a wide connotation that included antibiotics. Although numerous definitions have been proposed since then, none has proved completely satisfactory because of the need for additional explanations, e.g., with regard to statements such as "beneficial balance," "normal population," or "stabilization of the gut flora." In 1989, Fuller (Fuller, 1989) attempted to improve Parker's definition of probiotic with the

following distinction: “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.” This revised definition emphasized the requirement of viability for probiotics and introduced the feature of a beneficial effect on the host, which was, according to his definition, an animal. A similar definition was proposed by Havenaar and Huis in 't Veld (1992) “…mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora.” Probiotics are best known by the average consumer in relation to foods; in this contest the EU Expert Group on Functional Foods in Europe (FUFOSE) has defined them as “viable preparations in foods or dietary supplements to improve the health of humans and animals”. Salminen (1996) and Schaafsma (1996) broadened the definition of probiotics. According to Salminen, a probiotic is “a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host.” According to Schaafsma, “Oral probiotics are living microorganisms which upon ingestion in certain numbers exert health effects beyond inherent basic nutrition.” , In 2001, Schrezenmeir and Michael de Vrese proposed the following definition: “A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host”. In 2002, FAO/WHO has adopted the definition of probiotics as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/ WHO, 2002).

In the past decades studies in the area of probiotics have progressed considerably and significant advances have been made in the selection and characterisation of specific probiotic cultures and in the identification of the positive effects they have on health. Members of the genera *Lactobacillus* and *Bifidobacterium* are now mostly employed, but not exclusively, as probiotic microorganisms and a larger variety of probiotic foods are now available to the consumer.

The original assumption of Metchnikoff was that the dietary manipulation of the gut microbiota performed in order to increase the relative numbers of "beneficial bacteria" could contribute to the well being of the host. However he also stated that systematic investigations should be made on the relation of gut microbes to the age, and on the

influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body."

It is necessary to assess the efficacy and safety of probiotics and this constitutes an important part of their characterization for human use.

Microbes from many different genera are being used as probiotics. The most commonly used strains are members of the heterogeneous group of lactic acid bacteria; lactobacilli, enterococci and bifidobacteria.

4.2 Principal effects of probiotics on human gut

The mechanism of probiotic action is not totally known but different approaches could be developed. According to Fuller (1989) the probiotic effect of lactic acid bacteria and bifidobacteria may be expressed by three main mechanisms of action:

1. Suppression of pathogenic microorganisms in the intestinal tract by:

a) production of antibacterial substances including primary metabolites, such as lactic acid, acetic acid, carbon dioxide, diacetyl, acetaldehyde, hydrogen peroxide and bacteriocins; they are proteinaceous compounds with antimicrobial activities against other closely related bacteria;

b) competition for nutrients. In the large intestine, the competition is limited for some nutrients, in particular for specific carbohydrates and polysaccharides;

c) competition for adhesion receptors on the gut epithelium. Probiotic strains can adhere specifically or non-specifically. Specific adhesion takes place when a ligand on the bacterial cell binds to a receptor on the epithelial cell; this is commonly defined as a " lock and key " function. Non-specific adhesion is a more general phenomenon mediated by hydrophobic or electrostatic interaction and does not seem to have particular relevance in the colonisation of epithelia *in vivo.*

2. Alteration of microbial metabolism in intestinal tract:

a) increasing the activity of useful enzymes, *e.g.* β-galactosidase in the alleviation of lactose maldigestion;

b) decreasing the activity of some colonic enzymes such as nitroreductase and azoreductase known to have carcinogenic effects.

3. Stimulation of immunity: recent reports have shown that orally administered lactobacilli and bifidobacteria can improve immune status by increasing the circulating

and local antibody levels, the gamma interferon concentration, the macrophage activity and the number of natural killer cells (MacDonald and Monteleone, 2005). The inclusion of lactic acid bacteria and bifidobacteria as members of physiological indigenous microflora into the mucosa and the subsequent translocation to other organs is currently regarded as a crucial step for the development of the normal mucosal and systemic immunity.

4.3 Use of probiotics in pedriatrics

An increasing number of clinical trials have documented effects of ingestion of specific probiotics bacteria on the care of important infant diseases. The use of probiotics formula for infants older than 4 months of age has already been approved by the American Food and Drug Administration (FDA) and in particular *B. lactis* obtained the GRAS (*generally regarded as safe*) status. In addition some recent works have shown encouraging data about administration of *Bifidobacterium breve* strains in preterms and low birth weight infants (Li et al., 2004 and Wang et al., 2007) and a wide literature documentation reports clinical benefits with treatment of infant gastrointestinal disease with probiotics.

One of the best-studied clinical outcome with the use of probiotics bacteria has been acute diarrheal disease in infants. The majority of the studies have been included various species of lactobacilli and bifidobacteria, and by far, the most used have been *Lactobacillus rhamnosus* (LGG*), Lactobacillus reuteri* and *Bifidobacterium lactis* (Guandalini et al., 2000 and Weizman et al., 2005). The larger number of trials documents therapeutic use of probiotics as supplements early in the course of the disease and the most consistent effect reported is a reduction in duration of illness, while another part of literature examine the reduction in incidence of acute diarrheal disease after a preventive administration of probiotics and these studies documented reduction in incidence or severity of the illness (Saavedra and Tschernia, 2007). No study to date has documented an increase in diarrheal disease with any probiotic strain used. Moreover, several probiotics strains resulted effective in reducing the risk of antibiotic-associated diarrhea in newborns and children. A clinical trial, performed with 766 infants, indicated that concomitant treatment with probiotics, compared with placebo, reduced the risk of diarrhea from 28.5% to 11.9% (Szajewska et al., 2006).

Some recent works have described clinical trials conducted on preterm infants. The theoretical benefits of probiotics in preterm infants include the prevention of NEC. These initial studies are encouraging and demonstrate the efficacy of probiotics to re-establish the balance of the gut flora by increasing the number of bifidobacteria. The most used probiotics strains were *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium bifidum.* In all these works the oral administration of probiotics showed a significant reduction in NEC incidence and NEC-associated mortality in respect with placebo group (Bin–Nun et al., 2005 and Lin et al., 2008).

A new aspect of the application of probiotics in the pediatric field is the treatment against gas colics. A published study (Savino et al., 2007) examinated, for the first time, the modulation of intestinal microbiota of colickly infants by administering a probiotic strain. A cohort of 90 breastfed colickly infants was randomly assigned to treatment with the probiotic *Lactobacillus reuteri* and simethicone. This study evidenced that infants treated with *L. reuteri* had a significant reduction in crying compared to infants treated with simethicone. The hypothesis, therefore, that probiotic supplementation can provided a reduction of gas colic symptoms and a modulation of intestinal microbiota was demonstrated (Savino et al., 2007, Savino et al., 2010).

To conclude, other clinical trials have shown a great improvement in infants affected by atopic dermatitis after administration with probiotics formula, in these cases, the severity of skin manifestation was strongly reduced (Viljanen et al., 2005). Lower counts of bifidobacteria have been reported in atopic vs non atopic children preceding allergen sensitization. Therefore, bifidobacteria are hypothesized to more effectively promote tolerance against antigen, stimulating GALT immune response.

4.4 *In vitro* selection of probiotic strains

Although progress in probiotic research has been achieved over the past few years, not all of the available probiotic bacteria which are on the market have adequate scientific documentation. It should be desirable to understand the mechanisms that determine the nutritional and health benefits derived from products containing probiotic bacteria, and to use the most promising strains. The probiotic concept will only gain acceptance if these underlying mechanisms are elucidated. Consequently, it is necessary to establish

rational criteria for the screening and selection of candidate microorganisms and also to evaluate the efficacy of the selected strains or the food products in well-controlled human clinical trials.

Significant progress in legislation for the safety evaluation of probiotics has been made in USA, Canada, and Europe (EFSA, 2005a; HC, 2006; FAO/WHO, 2002); however, no unique standards are available. In the USA, microorganisms considered safe for human consumption are awarded the GRAS status by the 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. The QPS concept provides a generic assessment system for use within EFSA that in principle can be applied to all requests received for the safety assessments of microorganisms deliberately introduced into the food chain (EFSA, 2005b). EFSA has published a list of microorganism, which possess a known historical safety, proposed for QPS status (EFSA, 2007a). Although the FAO and WHO reports were mainly focused on foods enriched with probiotics, many of the recommendations, including the definition of probiotics, were approved at the Meeting of the International Scientific Association for Probiotics and Prebiotics in May 2002.

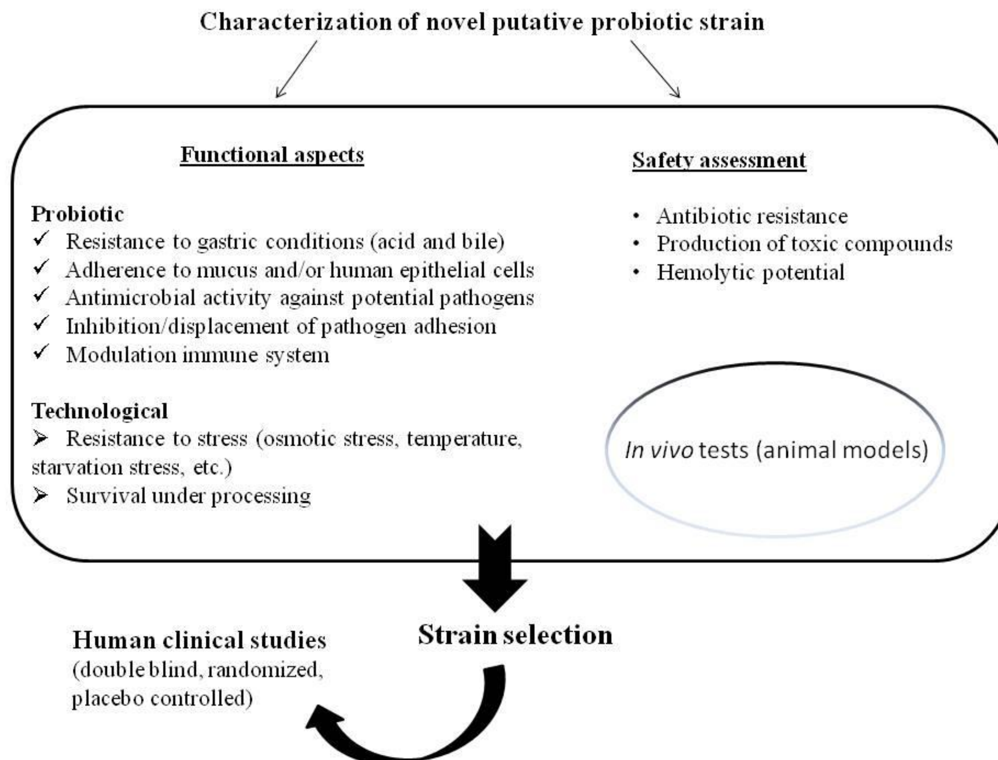


Figure 4 Procedure for the characterisation of novel strain with putative probiotic status.

The main steps for the selection of a novel probiotics strain are (**Figure 4**):

1. Strain identification;
2. Safety evaluation;
3. Fuctional characterization;

4.4.1 Strain identification

The first consideration is to identify and characterize the organism at the genus and species-level. Phenotypic tests may be useful to obtain a first tentative classification at the genus level but the identification results should in any case be confirmed by molecular methods. DNA-DNA reassociation is still considered as a reliable method for the delineation and description of a new bacterial species but it is impractical for the high cost and its complexity. Pattern- and sequence-based molecular methods provided actually a reproducible and easy methods thanks to the update of databases and data exchangeability. However, 16S rRNA does not allow a unequivocal separation of all the

taxa; for that reason it needs to be complemented by other molecular methods such as fingerprinting techniques: Amplified Fragment Length Polymorphism (AFLP), repetitive DNA element-PCR (rep-PCR) or Enterobacterial Repetitive Intergenic Consensus- PCR (ERIC-PCR). These techniques cold be used in association with sequencing of 23S rRNA , Internal Trascribed Spacer (ITS) elements and/or single copy genes (such as *groEL*, *recA*, *tuf*, *atpD*, *dnaK* and *grpE*).

Once the strain has been identified, a scientifically recognized name must be employed and the strains must be deposited in an internationally recognized culture collection.

4.4.2 Safety evaluation

As efficacy is inextricably linked to safety, any claims of health benefits for a probiotic require substantiation by scientific evidence.

The presence of antibiotic resistances and transferability of the antibiotic resistance genes are key factors in safety evaluation. In 2008, a decision of the FEEDAP Panel of EFSA updated the criteria used for the assessment of bacteria for resistance to antibiotics of human and veterinary importance (EFSA, 2008). The aim of this decision was to provide guidance for developing studies to show the potential of each bacteria strain to bear resistance and to transfer it. The basis of such evaluation starts with the determination *in vitro* of the minimal inhibitory concentration (MIC) for a relevant range of antibiotics of human and veterinary importance (**Table 3**). The detection of the MIC above the breakpoint levels for one or more antimicrobials required further investigations to make the distinction between acquired and intrinsic resistance; the microbiological breakpoints categorizing bacteria as resistant are expressed in **table 3**. According to the principle of FEEDAP, when a bacterial strain proves resistant to a specific antibiotic, while others species are normally susceptible to the same antibiotic, the applicant should evaluate the reason for such resistance. If an acquired resistance may be transferred or if known exogenous resistance genes are present, the probiotic strain is not considered suitable for use as food or feed additive

In addition, the determination of antibiotic resistance among probiotic microrganisms is affected by problems regarding the use of media, furthermore, MIC breakpoint values have been shown to be species specific and consequently they vary between species of the same genera.

From the evaluation of the current scientific data, it has been concluded that there is not a precise standard to enforce to assess the resistance of probiotic strains to antibiotics; further studies are needed.

Table 3 The Microbiological breakpoints used by EFSA 2008 categorising bacterial species as resistant (mg/l)

	Bifidobacterium	***Enterococcus***	***Pediococcus***	***Leuconostoc***	***Lactococcus lactis***	***Streptococcus thermophilus***	***Bacillus spp.***	***Propionibacterium***
Ampicillin	2	4	4	2	2	2	n.r.	2
Vancomycin	2	4	n.r.	n.r.	4	4	4	4
Gentamycin	64	32	16	16	32	32	4	64
Kanamycin	n.r.	512	64	16	64	64	8	64
Streptomycin	128	128	64	64	64	64	8	64
Erythromycin	0.5	4	1	1	2	2	4	0.5
Clindamycin	0.25	4	1	1	4	2	4	0.25
Quinupristin/dalfopristin	1	4	4	4	4	4	4	0.5
Tetracycline	8	2	8	8	4	4	8	2
Chloramphenicol	4	8	4	4	8	4	8	2

n.r. = Certain species are inherently resistant, and for these species MIC determination is not necessary

Safety assessment for new probiotic strains may also include the evaluation of the potential cytotoxic effects of the microorganisms on human cells. 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, for example toxicity studies of *Bacillus* probiotic strains have found no evident toxicity in lower animals such as mice and piglets (Sorokulova et al., 2008). Animal studies also present major disadvantages like the disagreement with the bioethical spirit of reducing animal testing in the EU, therefore, the need for a suitable cell culture model is to be consider paramount in order to avoid the use of a large number of animals.

As it has been formerly discuss in chapter 3, different kind of *in vitro* cell models of the gut are now available and they represent a reliable system for assessing the potential cytotoxity of probiotics strains (Cencič and Langerholc, 2010).

4.4.3 Functional characterization

In vitro tests of candidate probiotic strains, some of them summarized in **Table 4**, are thought to provide some insight for a more appropriate choice for *in vivo* functionality.

Table 4 Main *in vitro* tests currently used for the study of probiotic strains (from report FAO, 2002)

Resistance to gastric acidity
Bile acid resistance
Adherence to mucus and or epithelial cells and cell lines of humans and/or animals
Antimicrobial activity against potentially pathogenic bacteria
Ability to reduce pathogen adhesion to surfaces

Among the criteria used for the selection of probiotic strains, the most commonly employed is the survival in the stressful GIT conditions (low pH and high bile salts concentrations), the ability to transitory colonize the GIT, which is related with the adhesion to mucus and/or intestinal epithelium and the antimicrobial activity through the production of antimicrobial molecules or the ability to inhibit/displace the adhesion of pathogens. Several *in vitro* and *in vivo* tests are employed for the screening of these characteristics, although there is a lack of standardised or unified methodology for the assessment of probiotic functionality.

Chapter 5. The genus Bifidobacterium

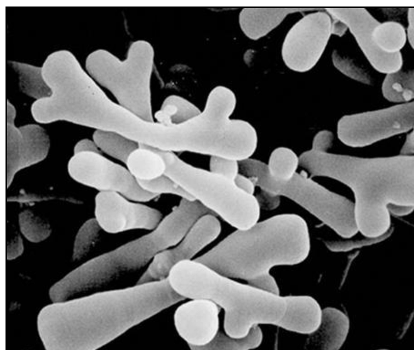


Figure 5 Scanning electron micrographs of *Bifidobacterium* spp.

5.1 Physiology and metabolism

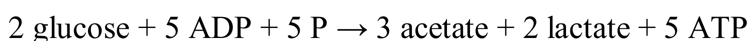
Bifidobacteria are Gram-positive polymorphic branched rods that occur singly, in chains or in clumps (**Figure 5**). They are non-spore forming, non-motile and non-filamentous. They are anaerobic : their sensitivity to oxygen changes in relation to the species and the different strains of each species. Bifidobacteria are chemoorganotrophs, having a fermentative type of metabolism. They produce acid but not gas from a variety of carbohydrates. They are catalase negative (with some exceptions). Their genome GC content varies from 42 mol% to 62 mol% (Biavati and Mattarelli, 2001).

The optimum temperature for growth is 37-41 °C, while no growth occurs below 20 °C and above 46 °C. Growth at 45 °C seems to discriminate between animal and human strains. Bifidobacteria are acid-tolerant microorganisms.

The optimum pH is between 6.5 and 7.0 and no growth is recorded below pH 4.5. Bifidobacteria are in fact acid tolerant but they are not acidophilic microorganisms.

Bifidobacterium spp. produce lactic and acetic acid from glucose.

The global equation is:



2 glucose + 5 ADP + 5 P → 3 acetate + 2 lactate + 5 ATP

This peculiar metabolic pathway is called “fructose-6-phosphate shunt” or “bifidus shunt”. The key enzyme of this pathway is fructose-6-phosphate-phosphoketolase, which is considered a taxonomic character for the identification on the genus level (Biavati and Mattarelli, 2001). Different species produce variable amounts of acetate, lactate ethanol and formate under the same conditions. The bifidobacteria utilize a great

variety of mono- and disaccharides as carbon sources and are able to metabolize also complex carbohydrates that are normally not digested in the small intestine. This feature should give an ecological advantage to colonizers of the intestinal environment where complex carbohydrates, such as mucin, are present either because they are produced by the epithelium of the host or because they are introduced through diet.

5.2 *Bifidobacterium* spp.

In 1900, Tissier observed and isolated in the feces of breast-fed infants a bacterium with a strange and characteristic Y shape and called it "*Bacillus bifidus*" (Tissier, 1899). This bacterium was anaerobic, Gram-positive and did not produce gas during its growth (Tissier, 1899). He proposed its inclusion in the family *Lactobacillaceae*. For a long time, bifidobacteria were included in the genus *Lactobacillus*. In the 8th edition of *Bergey's Manual of Determinative Bacteriology* bifidobacteria were classified for the first time in the genus *Bifidobacterium* and comprised eight species.

Nowadays, according to Taxonomic Outline of the Prokaryotes, the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, sub-class *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. The other genera belonging to this family are: *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia* and *Scardovia*.

At present the species included in the genus *Bifidobacterium* are:

Bifidobacterium adolescentis, *Bifidobacterium angulatum*, *Bifidobacterium animalis (with two subspecies B. animalis subsp. animalis and B. animalis subsp. lactis)*, *Bifidobacterium asteroids*, *Bifidobacterium bifidum (type species)*, *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium indicum*, *Bifidobacterium longum*, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum (with the two subspecies B. pseudolongum subsp. pseudolongum and B. pseudolongum subsp. globosum)*, *Bifidobacterium psychraerophilum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium thermacidophilum (with the two subspecies B. thermacidophilum subsp. thermacidophilum and B. thermacidophilum subsp. porcinum)*, and *Bifidobacterium thermophilum*.

5.3 Bifidobacterial population in the infant gut

The intestinal microbiota of breast-fed newborns is predominantly composed by bifidobacteria. In particular the most abundant *Bifidobacterium* species isolated from newborns gut are: *B. breve, B. longum* subsp. *infantis and B. longum* subsp. *longum. B. catenulatum, B. pseudocatenulatum, B. bifidum, B. dentium* have also been isolated from infant gut but they are not the dominant species (Biavati et al., 1984). However standard infant formula seems to give a more adult-like microbiota at the level of *Bifidobacterium* species such as lower level of *B. breve* and higher level of *B. catenulatum.*

In addition formula-fed newborns can be colonized by *B. adolescentis* from the mothers (Haarman and Knol, 2005). Furthermore, several studies reported differences in the levels of *Bifidobacterium* species between allergic and nonallergic infants with a more adult-like microbiota in allergic infants (He, 2001, Ouwehand et al., 2001).

5.4 Identification at the species level of the *Bifidobacterium* strains

Since some strains of the genus *Bifidobacterium* have been used for clinic and therapeutic purposes, and due to the growing industrial importance, it has become increasingly important to establish a precise classification scheme for the increasing number of bifidobacterial species.

The classical procedures for the identification of *Bifidobacterium* are based on cultivation-method approaches. The morphology can help in the identification at the genus level, but is not sufficient to recognize bifidobacterial species. However, one of the more practical approaches to the primary differentiation of bifidobacteria from related groups is based on identification by gas chromatography of the fermentation products, among which acetic acid generally predominates over lactic acid as the main final product. The most direct and reliable assignment of bacterial strains to the *Bifidobacterium* genus is based upon the demonstration, in cellular extract, of the presence of fructose-6-phosphase phosphoketolase, the key enzyme of bifidobacterial hexose metabolism.

During the last decade the development of molecular approach as based on sequence comparisons of DNA or RNA has provided a profound modification in the identification methodologies, moreover the availability of several whole genome

sequences has allowed significant progress in the identification of these bacteria and permitted various classification adjustments. In the past decades DNA-DNA hybridization methods were used to determine the belonging to a bacterial species, however this method is time-consuming and sometimes the results achived are questionable. During past years, therefore, the molecolar tools with regards to identification and classification methods were based on 16S rRNA gene as a molecular marker for deducing phylogeny in bacteria. The majority of the molecular tools for the identification of bifidobacterial species such as Amplified rDNA Restriction Analysis (ARDRA) (Ventura et al., 2001a), specie-specific primers (Matzuki et al., 1999, Ventura et al., 2001b) and denaturing gradient gel electrophoresis (DGGE) (Favier et al ., 2002) are all based on 16S rRNA sequence.

However, some bifidobacterial taxa have a very high degree of similarity or even possess identical 16S rRNA gene sequences such as *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium animalis* subsp. *lactis, Bifidobacterium longum* subsp. *longum* and *Bifidobacterium longum* subsp. *infantis, Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum.* To this end, new molecular approaches have been developed to ride over these taxonomic difficulties. In recent year, alternative genomic sequences have been used as molecular markers for the identification of bifidobacteria, such as *groEL* (Jian et al., 2001), *recA* and *tuf* (Ventura and Zink, 2003), *atpD* (Ventura et al., 2004), *dnaK* and *grpE* (Ventura et al., 2005a). Evolutionary study using single genes are popular because they allow quick and unequivocal results, however there are not still a complete sequence database for such genes. The criterion used to select new potential genes are not only their conservation in the genome they should not be susceptible to horizontal transfer events, G+C skew, dinucleotide frequency and codon usage analyses. The above-mentioned genes have been already used to investigate the phylogeny of bifidobacterial species and for each gene a tree was calculated in order to evaluate the overall compatibility between the different trees. Generally, these genes tested showed a discriminatory power as compared with the 16S rRNA gene, however, single gene tree may not adeguately reflect phylogenetic relationships, because of the possibility of horizontal transfer events; consequently a phylogenetic tree using multigene conconcatenation approach reveals an increase discriminatory power and a most reliable picture of evolutionary relationships (Ventura et al 2006b).

Another technique wich allow phylogenetic and typing characterization of *Bifidobacterium* strains is the internal transcribed spacer (ITS) sequence analysis.
Recently it was demonstrate that ampliflied ribosomal DNA restriction had powerful potential in the discrimination of various bifidobacteria to the species level. Enterobacterial Repetitive Intergenic consensus (ERIC)-PCR involves the use of oligonucleotides targeting short repetitive sequences dispersed throughout various bacterial genomes. Their location in bacterial genomes allows a discrimination at the genus, species and strain level based on their amplification pattern fingerprinting. This molecular approaches for a identification of bifidobacterial species was carried out by Ventura et al., 2003. This ERIC-PCR approach generated specie specific patterns for all investigated species of *Bifidobacterium*. This technique is a rapid, reproducible, and easy-to-handle molecular tool to enable highly specific detection and identification of bifidobacterial species within a mix of other bifidobacteria or in pure culture concentrates..
ERIC-PCR can be a very useful tool in the rapid detection of various bifidobacterial species in commercial products since it does not require any bacterial cultivation step. So far, ERIC-PCR approach is evaluated for directly tracing bifidobacteria in dairy products or in infant formulae containing only bifidobacteria and not for any other microorganisms without any purification steps.

Chapter 6. Prebiotics

6.1 The prebiotic capacity of different oligosaccharide compounds

Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). For a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host (Scantlebury-Manning and Gibson, 2004). The effects of dietary fiber on upper and lower gastrointestinal tract are shown in **Table 5**. One of the strongest health benefits proposed for prebiotics is the amplification of the resistance against invading gastrointestinal pathogens that is directly linked to the selective stimulation of probiotic microorganisms (Gibson et al., 2004). The consumption of prebiotics has also been associated to the reduction of the serum lipid concentration, throught a mechanisms involving modulation of hepatic lipogenesis probably by short chain acids adsorbtion from the gut. Furthermore some fructooligosaccharides have been linked to stimulate adsorption and retention of several minerals and to improved mineralization of bone, particularly magnesium, calcium and iron.

Most identified prebiotics are carbohydrates and oligosaccharides normally occurring in human and animal diet, with different molecular structures; dietary carbohydrates such as fibers, are candidate prebiotics, but most promising are non-digestible oligosaccharides (NDOs). NDOs which meet the critical point of the definition are fructooligosaccharides (FOS, oligofructose, inulin), galactooligosaccharides (GOS) or transgalactooligosaccharides (TOS), and lactulose; however a large number of other NDOs, to which less rigorous studies have been so far applied are gluco-oligosaccharides, glycololigosaccharides, lactitol, isomaltooligosaccharides, maltooligosaccharides xylooligosaccharides, stachyose, raffinose, and sucrose oligosaccharides (Patterson and Burkholder, 2003). Furthermore recent studies demonstrated the increasing interest in the capability of arabinogalactans and partially hydrolysed guar gum (PHGG) to stimulate the colonic growth of bifidobacteria and

lactobacilli. Arabinogalactans 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. Arabinogalactans derived from the larch tree are commercially available as fiber ingredients and they are considered as nondigestible soluble dietary fibers.

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 is a polysaccharide composed of the sugars galactose and mannose (galactomannan) (Alam et al., 2000).

Table 5 Intestinal functions assigned to prebiotics.

Dietary fibers and gastrointestinal functions	
Effect on upper GI tract	Resistance to digestion
	Retarded gastric emptying
	Increased oro-caecal transit time
	Reduced glucose absorption and low glycaemic index
	Hyperplasia of the small intestinal epithelium
	Stimulation of secretion of intestinal hormonal peptides
Effect on lower GI tract	Acting as food for colonic microbiota
	Acting as substrates for colonic fermentation
	Production of fermentation end products (mainlt SCFAs)
	Stimulation of saccharolytic fermentation
	Acidification of the colonic content
	Hyperplasia of the colonic epithelium
	Stimulation of secretion of colonic hormonal peptides
	Bulking effect on stool production
	Regularization of stool production (frequency and consistence)
	Acceleration of caeco-anal transit

6.2 FOS, fructooligosaccharides

FOS are natural food ingredients commonly found in varying percentages in dietary foods. They are present in > 36.000 plant species. The number of monosaccharides present in the molecule varies from 3 to 10. They are present as storage carbohydrates,

together with inulin, in a number of vegetables and plants including wheat, onion, bananas, garlic and chicory. These oligosaccharides are manufactured by two different general methods, which result in slightly different end products. In the first method they are produced from the disaccharide sucrose using the transfructosylation activity of the enzyme β-fructofuranosidase (or fructosyltransferase). The second method is instead the controlled enzymatic hydrolysis of the polysaccharide inulin.
For what concern the synthesis of FOS from sucrose, the enzyme source can be divided into two classes: one comprehends plants such as asparagus, sugar beet, onion, Jerusalem artichoke etc.; the other consists of enzymes of bacterial and fungal origins such as *Aspergillus* spp., *Aureobasidium* spp., *Arthrobacter* spp., *Fusarium* spp.. The production yield of FOS using enzymes originated from plants is low and mass production of enzyme is limited by seasonal condition, therefore industrial production depends chiefly on fungal enzymes from either *Aureobasidium* spp. or *A. niger*. Moreover these enzymes are more stable than those of plants.
For the production, a high concentration of the substrate (sucrose) is required for efficient reaction. The FOS formed in this process contain between two and four β(1→2)-linked fructosyl units linked to a terminal α-D-glucose residue. The oligosaccharides are named: 1-kestose (GF2, glucose-fructose2), 1-nystose (GF3) and 1F-fructosylnystose (GF4) (**Figure 6**).

FOS, together with inulin, are the most studied and well established prebiotics. It has been demonstrated that intake of FOS reduces significantly the count of *Bacteroides* spp. and clostridia. The increase in bifidobacteria is accompanied with other beneficial effects such as: modulation of intestinal functions, increase of stool weight, decrease of faecal pH (probably linked to the suppression of the production of putrefactive substances in the colon), modulation of cholesterol levels and modulation of mineral metabolism (Roberfroid, 2005).

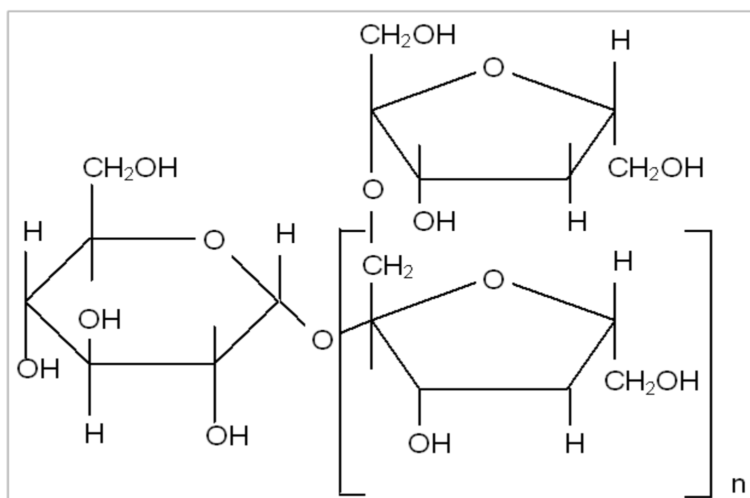


Figure 6 General structure of sucrose derived FOS.

6.3 Inulin

Inulin is a polydisperse β(1→2) fructan. A glucose molecules typically resides at the end of each fructose chain and is linked by an α (1→2) bond to sucrose, but this is not necessary. Different fructans compounds are included under the same nomenclature, and they are both a mixture of oligomers and polymers that are characterized by a different degree of polymerization (DP). The chain lengths of these fructans range from 2 to 60 units with the average DP ~ 10. The unique aspect of the structure of inulin is its β(1→2) bonds. These linkages prevent inulin from being digested like a typical carbohydrate and are responsible for its reduced caloric value and dietary fiber effects.
The DP of inulin and the presence of branches are important properties that influence its functionality strikingly There is a strict distinction between inulin of plant and bacterial origin. The DP of plant inulin is low (DP < 60) in respect of bacterial inulin and varies according to the plant species. Moreover plant inulin are considered to be linear molecules with a very small degree of branching (1-2%). O the contrary bacterial inulin has a DP that varies from 10,000 up to 100,000 and it is highly branched (15%).
Inulin is present in significant amounts in several fruits and vegetables that have been analyzed, and in different plant species there is a great diversity of inulin types.
Inulin content ranges from less than 1 up to some 20% of fresh weight. In banana, for example, 100% of oligomers have a DP< 5, but in salsify (*Tragonopon porrifolius*), 75% have a DP ≥5. In onion, DP ranges from 2 to 12, in chicory it ranges from 2 to 65, in globe artichoke 96% have a DP> 5 and 87% of polymers have a DP≥40.

Inulin, with different chain lengths, is fermented at different rates according to their DP. Inulin with a low DP is fermented in the proximal part of the colon. Its intensive fermentation modifies drastically the composition of the intestinal microbiota (bifidogenic effect) in the more proximal part of the large intestine. The long chain inulin (HP-inulin), on the other hand, which is fermentedat a slower rate, is able to reach more distal parts of the colon. In this part of the intestine, easily fermented carbohydrates are scarce, so bacterial catabolism shifts towards proteolysis, which results in the production of toxic putrefactive products. HP-inulin is able to reduce the proteolytic activity in favour of a beneficial saccharolytic activity in the distal parts of the colon.

Several experiments have demonstrated the increase of *Bifidobacterium* population after inulin intake in the gastrointestinal tract and also the growth of certain lactobacilli. Bifidobacteria have an inducible β-fructofuranosidase enzyme able to hydrolyse the β(2,1) glycosidic linkages between the fructose moieties (Rossi et al., 2005, Kolida and Gibson, 2007).

6.4 GOS, galactooligosaccharides

GOS are manufactured from lactose using the transgalactosylase activity of β-galactosidase. They are therefore often referred as transgalactosylated oligosaccharides (TOS). This enzyme is a hydrolase enzyme and works by transferring galactose from lactose to water. Under condition of high lactose concentration, the enzyme utilises lactose as an alternative acceptor to water resulting in the formation of galactooligosaccharides. A variety of enzyme reactor configurations based upon free or immobilised β-galactosidases have been used to produce these NDOs (Rastall and Gibson, 2002).

The transgalactosylation reaction leads to the formation of a mixture of oligosaccharides varying from DP 3 to DP 6 (DP, degree of polymerisation), with the average containing 3-4 sugar moieties. The general structure of TOSs is: β-D-gal-(1→6)-[β-D-gal]n-(1→4)-α-D-glu (**Figure 7**).

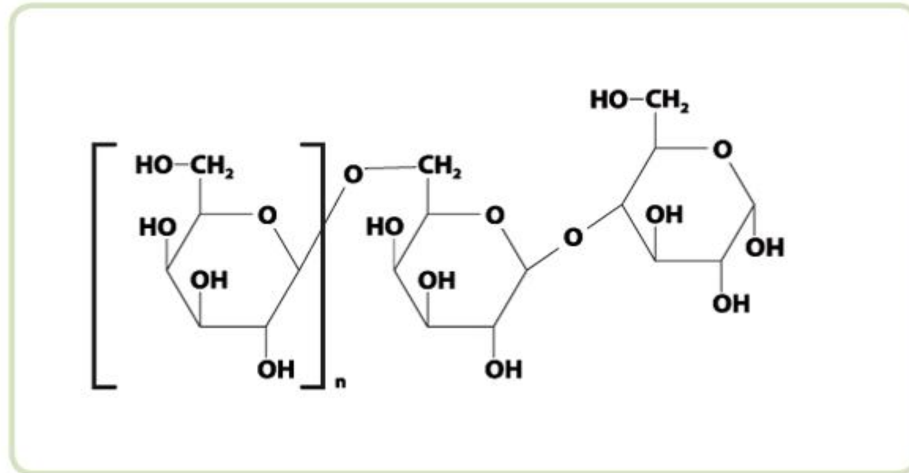


Figure 7 Chemical structure of galactooligosaccharides

The linkages between the galactose units, the efficiency of transgalactosylation, and the components in the final products depend on the enzymes and the conditions of the reaction. Using β-galactosidases derived from *Bacillus circulans* or *Cryptococcus laurentii*, the glycoside bonds between two galactose units are mainly β(1→4) bonds (4'-GOS). While using enzymes from *Aspergillus oryzae* or *Streptococcus thermophilus* glycoside bonds are mainly β(1→6) (6'-GOS). In standardized large scale production using the enzyme from *B. circulans*, more than 55% of the lactose is converted to GOS. The lactose used as substrate for GOS production is usually purified from cow's milk whey. The main products are trisaccharides, namely 4'- or 6'-galactosyllactose and also longer oligos (≥ 4 units).

The indigestibility of GOS in vivo has been demonstrated, GOS resists digestion and absorption in the small intestine and reaches the caecum and colon, where they are fermented by the colonic bacteria. 4'-Galactosyllactose is selectively utilized by all the *Bifidobacterium* strains tested compared with lactulose and raffinose whose specificity is less remarkable. But also strains of other genera are able to use GOS, such as strains of *Lactobacillus* and *Bacteroides*. However, the utilisation of NDOs by bifidobacteria is usually mediated by the hydrolyzing enzymes they produce, and many strains produce glycolytic enzymes which hydrolyze a wide variety of monosaccharide units and different glycoside bonds. Other enteric bacteria, on the contrary, have enzymatic activities that are less varied and with a weaker activity (Sako et al., 1999). *In vitro* fermentations with human faecal or rat caecal microbiota indicate that GOS increases

the production of acetate and propionate. Follow on studies have addressed the galactooligosaccharides in respect to GOS fermentation by pure batch cultures. It has been demonstrated that these carbohydrates are readily fermentable by bifidobacteria, some but not all strains of *Bacteroides*, lactobacilli and *Enterobacteriaceae* but not by eubacteria, fusobacteria, clostridia, and most strains of streptococci (Gibson and Roberfroid, 1999).

GOS have demonstrated positive effects on calcium absorption and have prevented bone loss in some animal research. In preliminary studies, GOS have shown some ability to lower triglyceride levels. GOS are now used as sweeteners by themselves, especially in fermented milk products, breads, jams, etc. For example GOS in bread are not broken down by yeasts and render the bread excellent in taste and texture. Fermented milk products containing probiotic bacteria with added GOS are commercially available in Japan and in Europe. Baby foods are promising fields of application of GOS.

6.5 Human milk oligosaccharides: the prebiotic effect of human milk

The characteristic composition of the intestinal microbiota of breast-fed neonates is in part due to the presence of oligosaccharides (HMO) in human milk. These HMO are resistant to digestive processes and thereby reach the colon, where they exert a prebiotic effect. Cow's milk, which is commonly used in the preparation of infant milk formulas, and human milk have significant differences.

HMO are one of the most important component in human milk, in contrast, these oligosaccharides are present only in small amounts in cow's milk. HMO are synthesized in the mammary gland by the action of specific glycosyltransferase by the sequential addition of monosaccharide units to the lactose molecule; the monosaccharides building blocks are glucose, galactose, N-acetylglucosammine, fucose and sialic acid (**Figure 8**). Over the years the prebiotic effect of HMO has been confirmed and *in vitro* fermentation studies clearly demonstrated that bifidogenic effect of maternal milk is mainly due to the "non protein fraction" and that HMO 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 HMO as a sole source of carbon and energy and this is the confirmation that bifidobacteria can utilize complex

carbohydrates such as HMO. Recent studies focused on the molecular mechanisms underlying the promotion of human milk to specific development of intestinal bifidobacterial community, the identification of genes expressed by *B. breve* strains, upon HMO stimulation, rapresented the preliminary insight to understand the molecular mechanisms governing the initial stages of bacterial colonization in newborns (Turroni et al., 2011). Although some papers reported the isolation of bifidobacteria from human milk (Martin et al., 2003), an alternative hypothesis is that bifidobacteria are introduced into human milk throught newborn-mother contacts.

Another characteristic substance of human milk is lactoferrin which 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 extimated 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 and Gabrielli, 2008).

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.

In conclusion, 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

GIT of breast-fed neonates, the relationship between HMO and the development of bifidobacteria represents a typical example of this situation.

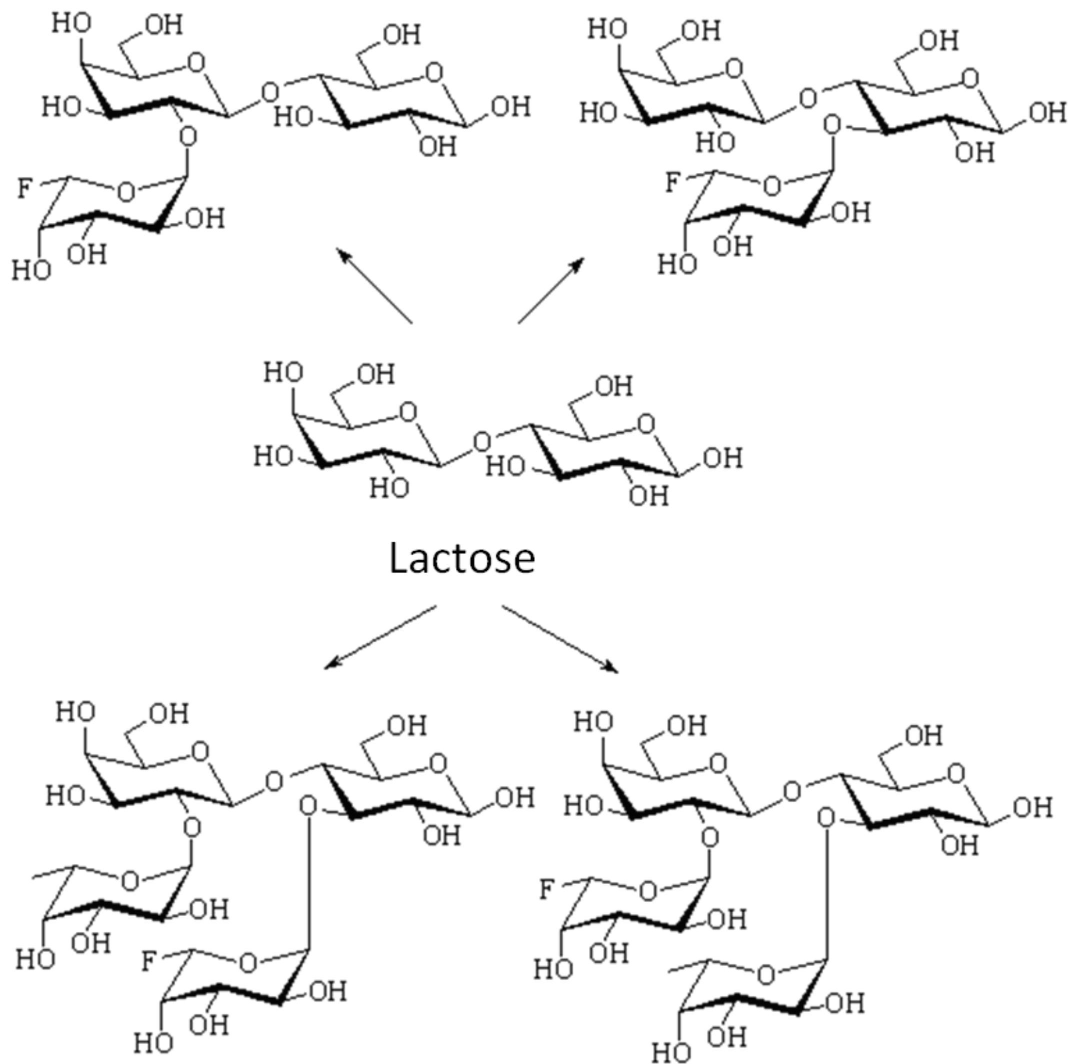


Figure 8 Chemical structure of human milk oligosaccharides

Chapter 7. Molecular approaches to study the gut microbiota

7.1 Different molecular methods for studying the microbiota

It is now generally accepted in microbial ecology that cultivation-based approaches provide an incomplete picture of microbial diversity in the gastrointestinal tract because only a minority of microbes can be obtained in culture. Therefore the application of molecular approaches, especially those focused on 16S ribosomal RNA sequence diversity, have become popular as they enable researchers to bypass the cultivation step. These approaches have provided considerable information about microbial ecosystems, including the GI tract (Zoetendal et al., 2004).

Studies on human microbiota, by both culture based (Harmsen et al., 1999, Marteau et al., 2001), and culture-independent (Haarman and Knol., 2004, Penders et al., 2006, Scanlan et al., 2008) methods, have indicated that this environment is dominated by obligate anaerobes, but a diverse range of species have been detected. The traditional culture-based methods of assessing mammalian gastrointestinal tract community structure are extremely laborious, and it has been estimated that only 10–60% of total bacteria from this environment are able to be cultured.

Non-culture methods for assessing gut microbial ecology (reviewed in Zoetendal et al., 2004), such as the construction and analysis of 16S rDNA clone libraries (Wang et al., 2005), for example, have been instrumental in the discovery of new intestinal bacterial groups. Molecular indices of diversity, such as the community fingerprinting tools DGGE (Favier et al., 2002), T-RFLP (Sakamoto, 2004), have also provided insight into human gut microbial ecology. Although these procedures have proved useful for detecting community structure shifts, with the exception of fluorescent in situ hybridization- based studies (Kalliomäki et al., 2008), they have the drawback that they are typically not quantitative. Real-time PCR, on the contrary, can be quantitative as the number of target gene copies in DNA directly extracted from an environmental sample can be determined. Using group-specific primer sets, the abundance of a particular gene marker for a defined group in the community can be estimated by comparison to a standard curve (Penders et al., 2005).

7. 2 Real-time PCR

In real-time or quantitative PCR a targeted DNA molecule is simultaneously amplified and quantified. Two common methods for detection of products in q-PCR are the use of fluorescent dyes that intercalate with ds DNA fragment and the use of fluorescently labelled oligonucleotides. By observing the point where the fluorescence crosses a threshold level, or crossing point value or Cp value (depending on the equipment, also known as a Ct value), a cycle number can be acquired for samples with different initial DNA concentrations. If the initial concentration is high, the threshold level will be crossed earlier than when the initial concentration is low (Figure 9). By measuring the Ct value for samples with known concentrations, standard curves can be made that can then be used for absolute quantification. The standard curve that is created prior to quantification of unknown samples gives important information about two parameters. First, it shows the detection window, or the range over which data points can be acquired. It is, however, important to notice that a linear relationship is used for quantification, and that sometimes not all points (especially at the window borders) fit a linear relationship (figure 6.2). That is why a distinction can be made between the detection window (i.e., the window over which detection is obtained) and the linear range of amplification (i.e., the window over which a linear relationship of the standard curve can be obtained). The second parameter that can be derived from the standard curve is the amplification efficiency (AE) through the following equation: $AE = (10^{(-1/slope)}) - 1$. When the theoretical optimum of a target doubling in each cycle is reached, the slope of the standard curve will be –3.32 and the value of AE will be 1.00. The AE can be used in several ways. First of all, deviations from the optimal value of 1.00 indicate that the PCR is not performing optimally, either because of inhibition or because of a suboptimal PCR setup. Therefore, the AE is an excellent tool with which to perform PCR optimization. Unfortunately, there seems to be no consensus yet in the scientific community about the correct way to analyze quantitative data and to create standard curves for real-time PCR. Most published data show standard curves constructed of one data set whereas others analyze and use multiple data sets to calculate the AE (Wolffs and Rådström, 2006).

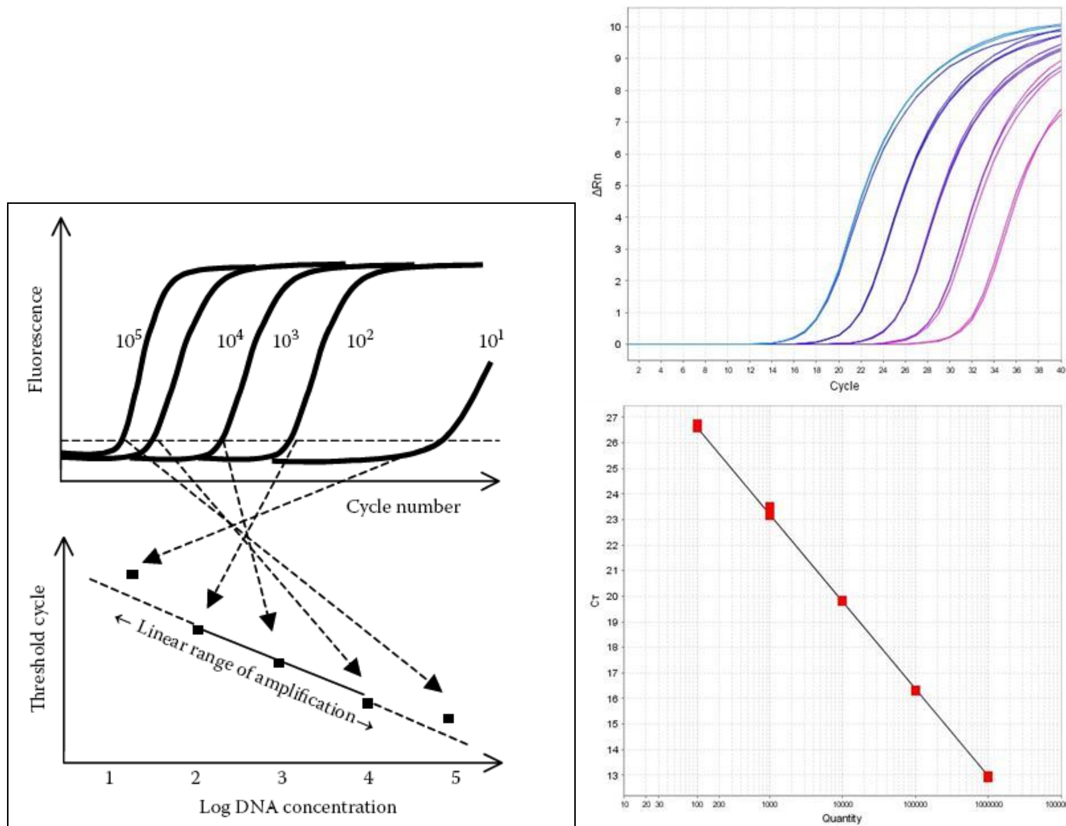


Figure 9 Schematic overview of the generation of a standard curve used for real-time quantitative PCR (Walffs and Rådström, 2006).

Two different approaches are possible in real-time PCR: nonspecific fluorescent dyes and labeled probes.

The standard method for nonspecific real-time detection of PCR amplicons is use of fluorescent double-stranded (ds)DNA intercalating dyes such as SYBR Green™ I or SYBR Gold™. Both of these commercial dyes are DNA minor groove binding dyes that fluoresce after interacting with dsDNA (**Figure 10**).

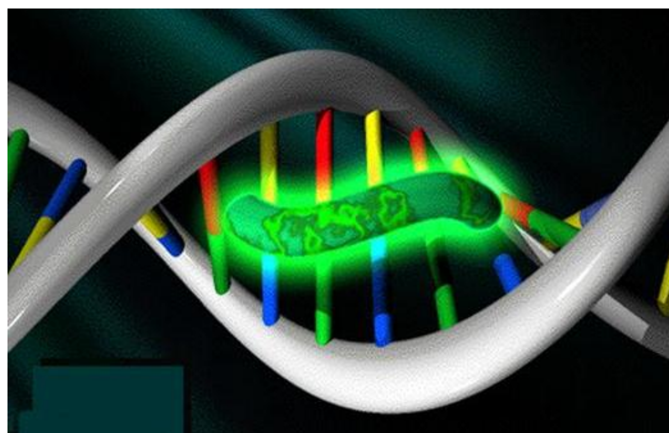


Figure10 Interaction of SybrGreen intercalating dye with double-stranded DNA and subsequent fluorescence under appropriate wavelength. The interaction is not sequence-specific.

Most real-time PCR instruments are programmed to read near the emission and excitation wavelength spectrum of SYBR Green™ (495 and 537 nm, respectively). This dye is very light sensitive, degrading quickly following dilution to working concentrations, but when fully active, allow the user to obtain real time fluorescence emission data (relative fluorescence units on the y-axis of a plot) as a function of cycle number on the x-axis. Since relative fluorescence units for each sample are plotted during the exponential phase of amplification, results are quantitative and thus useful for determining copy number and genome equivalents from template DNA obtained from different complex matrixes such as food and fecal samples. SYBR Green™ I has been used as an alternative to ethidium bromide for staining DNA in agarose gels, but it is also useful for real-time PCR detection assays, such as quantification of pathogen in humans, animal and food products. Due to the logistical difficulty in optimizing real time-PCR assays, the approach has limited potential for large-scale applications, particularly in light of many of the real-time chemistries. In addition to simply quantitative detection of target pathogenic or spoilage bacteria in foods, intercalating dyes such as SYBR Green™ I allow the system to discriminate among amplicons in a multiplex PCR reaction by using melt curve analysis. The melt curve analysis allows also to detect non-specific amplification, such as primer-dimers. This approach consists in a slow and continual heating to 95°C while monitoring fluorescence over time. Since each amplicon of a varying length and/or GC content will melt at a slightly different temperature, fluorescence will decrease incrementally according to the population of products in the reaction tube (Figure 11).

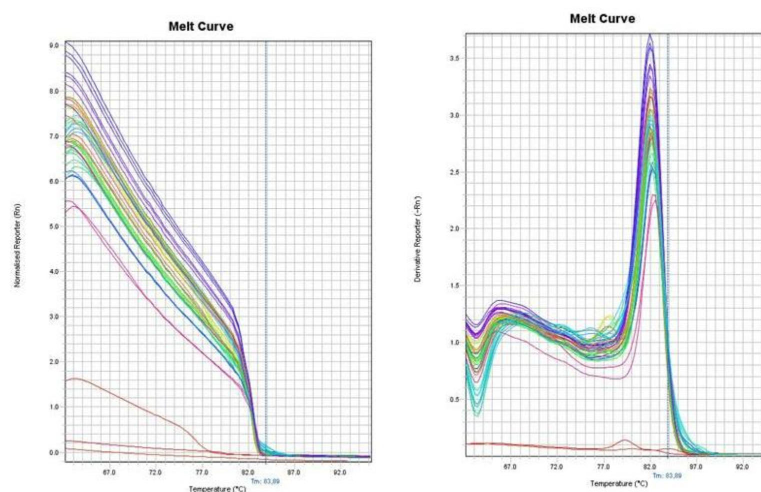


Figure 11 Example of a melting curve and its derivative.

A diverse array of fluorescently labeled probes are in use clinically and industrially for sequence-specific detection of target DNA or RNA. The primary category of these involves fluorescence resonance energy transfer (FRET) between a specific fluorophore and a quencher group. Perhaps the most widely used FRET conjugate pair for real-time PCR assays includes the fluorophore FAM (fluorescein) and the quencher tetramethylrhodamine (TAMRA). The resonance energy from the fluorophore is passed to the appropriate quenching moiety, and if in close proximity (as described below for specific primer and probe regimes), generates low levels, if any, detectable fluorescence as measured by a PCR cycler with fluorimeter capabilities. If separated or alone in solution, the fluorophore will not be quenched and the resonance energy will be emitted as a detectable fluorescent signal at the appropriate wavelength. Depending on the format of the PCR assay, the signal generated will be directly correlated with the amount of target DNA present or amplicon concentration. Regardless of the specific means in which the fluorophore/ quenching pair is applied, the basis remains the same, and includes the added advantage of sequence specificity that dsDNA intercalating dyes do not offer. One of the earliest uses for the FRET-based probe approach was the 5'-nuclease (TaqMan) assay, first described as a radioisotopic system, but soon modified to be based on fluorogenics. The 5'-nuclease activity incorporates a target gene-specific primer set and a dual-labeled probe that will hybridize to a region on one of the template strands within the primer annealing sites. During the extension phase of a PCR cycle, the 5'-3' exonuclease activity of Taq-polymerase will cleave the 5' fluorophore from the terminal end of the hybridized probe, separating it from the quenching moiety, eliciting fluorescence at a specific wavelength (**Figure 12**). Depending on the instrument being used for real-time detection, the investigator may choose to use multiple TaqMan primer and probe combinations in the same reaction tube for multiplexing, with each being detected in a unique optical channel at the respective wavelength.

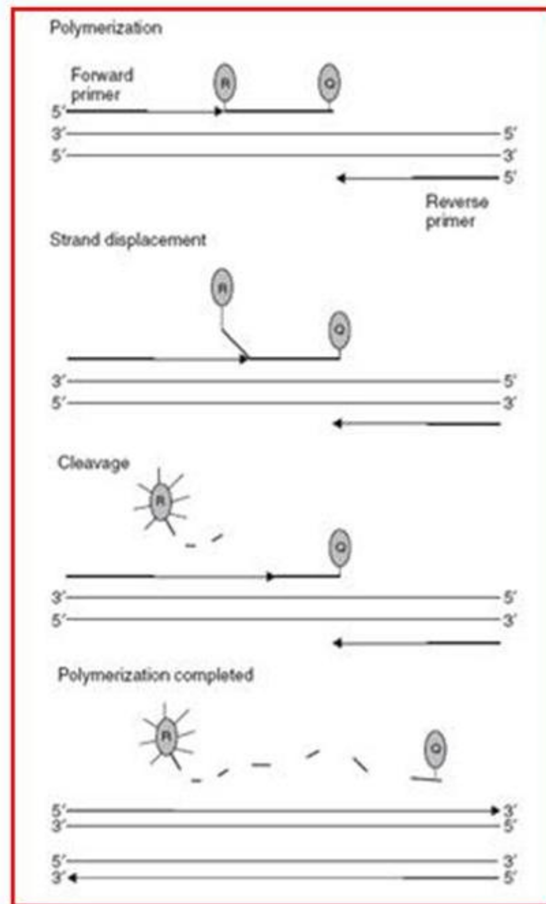


Figure 12 Mechanism of TaqMan 5' nuclease assay for real-time detection of PCR products using FRET-labeled probe internal to the sequence-specific primers. R denotes the reporter dye while Q represents the quenching moiety.

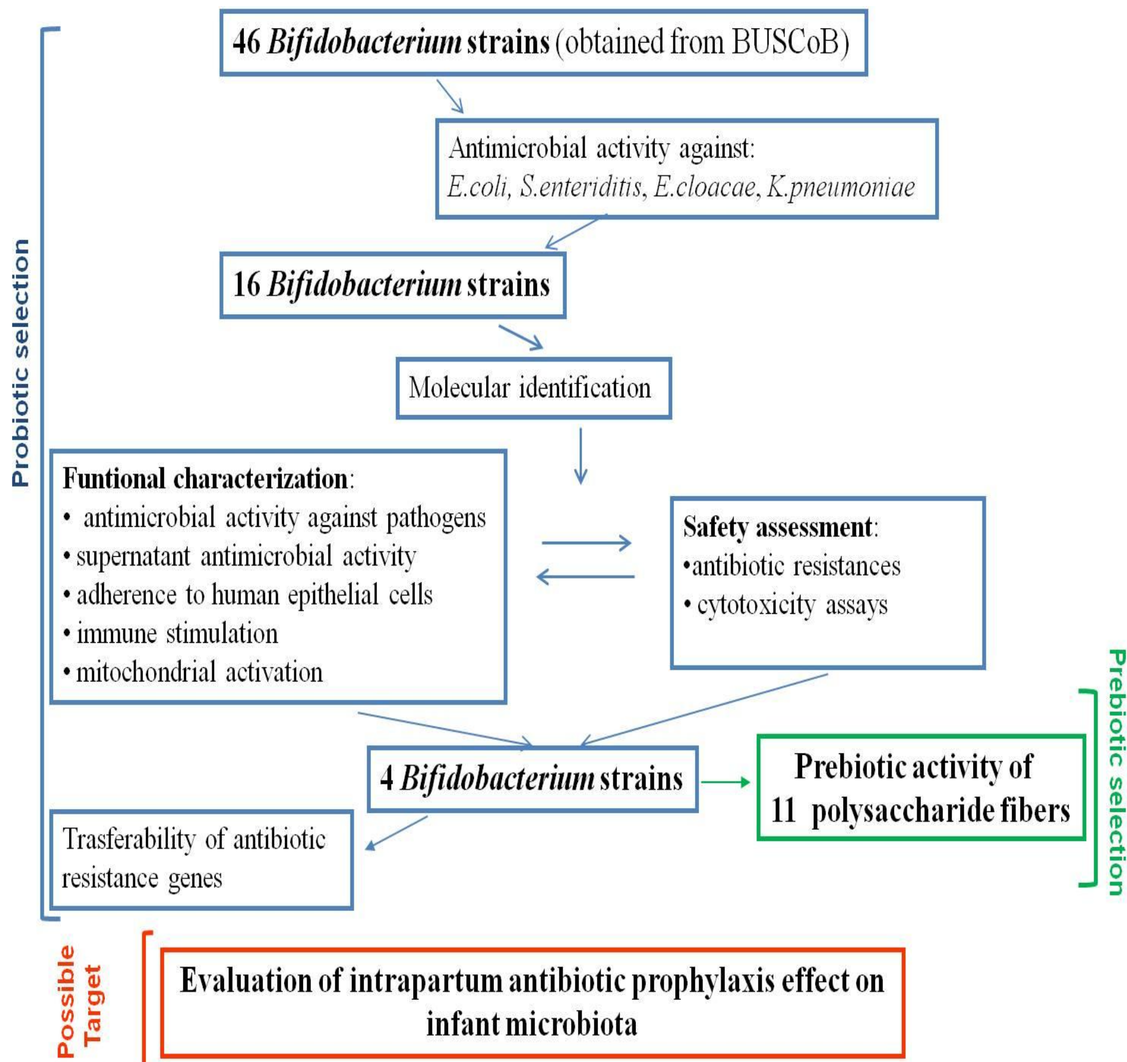
PART 2: AIM OF THE WORK

Bifidobacteria are the major components of the microbiota of infants fed exclusively with breast milk and are commensal bacteria of the large intestine of humans and animals. They are widely used as probiotics for therapeutic purposes considering their capabilities of colonizing the gastrointestinal tract and their long history of safe-use. Recent results evidenced that probiotics may be also useful for the treatment of minor gastrointestinal problems of newborns such as colics the daily administration of *L .reuteri* DSM 17938 in early breastfed infants was found to improve symptoms of infantile colics (Savino et al., 2010). No studies have been presented up to know on the possibility of using *Bifidobacterium* spp. strains for this enteric disorder, although, differently from *Lactobacillus* spp., *Bifidobacterium* spp. systemic infections upon administration in infants have never been reported.

The aim of this research was the selection of probiotic strains belonging to the *Bifidobacterium* genus to be used on newborns for the treatment of enteric disorders with a special attention on colics. The selection of the strains has been done among 46 *Bifidobacterium* strains, mainly deriving from human faeces, considering their capability of inhibiting the growth of pathogens typical of the newborn gastrointestinal tract and the evaluation of the basic safety properties according to the EFSA guidelines. In addition, a study performed in collaboration with the University of Maribor has evaluated *in vitro* the cytotoxic effect of the selected strains and their ability to adhere to non tumorigenic gut epithelial cell lines; the capability of the selected strains of stimulating the metabolic activity and the immune response of gut cells has also been examined. The formulation of a synbiotic product with an appropriate prebiotic fiber capable of supporting the growth of the selected *Bifidobacterium* strains was also considered in this study. The last phase of the work has been dedicated to the evaluation of the gut microbial diversity in newborns whose mothers has been subjected to antibiotic therapy a few hours before the delivery because of a *Streptococcus* type B infection. These newborns can represent a possible target for the probiotic strains selected in this work.

PART 3: MATERIALS AND METHODS

Chapter 8. Study design



A design of the whole study is preseted in this scheme. The work performed can be divided into three sections:

- Probiotic selection, described in chapter 9 and chapter 12 for Material and Methods abd results, respectively (Selection and characterization of *Bifidobacterium* strains);
- Prebiotic selection, described in chapter 10 and chapter 13 (Evaluation of the most effective prebiotic fiber);
- Possible target evaluation, described in Chapter 11 and chapter 14 (Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn microbiota).

Chapter 9. Selection and characterization of Bifidobacterium strains

9.1 *Bifidobacterium* strains and culture conditions

46 strains of *Bifidobacterium* spp. were included in this study; the majority of them derives from infant faeces and belong to five different species (*B. bifidum*, *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. adolescentis* e *B. pseudocatenulatum*) (**Figure 5**).

In **table 6** are reported all the *Bifidobacterium* strains and their original habitat. Forty-two of them were obtained from the Bologna University Scardovi Collection of Bifidobacteria (BUSCoB), available at the University of Bologna, whereas 4 were from the American Type Culture Collection (ATCC 15697, ATCC 15707, ATCC 15708, ATCC 27917). Thirty-six of the BUSCoB strains have been previously characterized with phenotypic analyses and by means of the electrophoretic pattern of transaldolase and 6-phosphogluconic dehydrogenase (Scardovi et al., 1979). The remaining 6 strains (B7710, B7740, B7840, B7947, B7958, B8452) were isolated from preterm newborn faeces and characterized as members of the *Bifidobacterium* genus by means of phenotypic analyses and the fructose 6-phosphate phosphoketolase assay (unpublished results). *Bifidobacterium* strains were cultivated in Tryptone, Peptone, Yeast extract medium (TPY prepared according to Biavati and Mattarelli 2006, see **table 7**) and incubated at 37 °C under anaerobic conditions using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany).

Table 6 List of the 46 *Bifidobacterium* spp. strains used in this study and their original habitat

Species	Strain	origin
B. bifidum	B1968	infant feces
B. bifidum	B2009	infant feces
B. bifidum	B2531	infant feces
B. bifidum	B2091	infant feces
B. breve	B2274	infant feces
B. breve	B2021	infant feces
B. breve	B632	infant feces
B. breve	B1501	infant feces
B. breve	B2150	infant feces

B. breve	B2142	infant feces
B. breve	B2228	infant feces
B. breve	B626	infant feces
B. breve	B633	infant feces
B. breve	B2136	infant feces
B. breve	B2023	infant feces
B. breve	B2195	infant feces
B. breve	B2210	infant feces
B.longum subsp. *infantis*	B1412[a]	infant feces
B.longum subsp. *infantis*	B651	infant feces
B.longum subsp. *infantis*	B1915	infant feces
B.longum subsp. *infantis*	B1860	infant feces
B.longum subsp. *infantis*	Re 6	infant feces
B.longum subsp. longum	B1629	infant feces
B.longum subsp. longum	Re11	infant feces
B.longum subsp. longum	Re12	infant feces
B.longum subsp. longum	B2101	infant feces
B.longum subsp. longum	B1975	infant feces
B.longum subsp. longum	B1482	infant feces
B.longum subsp. longum	B2327	infant feces
B.longum subsp. longum	B2212	infant feces
B.longum subsp. longum	B2192	infant feces
B.longum subsp. longum	B2055	infant feces
B.longum subsp. longum	B1993	infant feces
B.longum subsp. *longum*	B1996	infant feces
B. adolescentis	B7311	adult feces
B. adolescentis	B7162	adult feces
B. pseudocatenulatum	B1279	infant feces
Bifidobacterium spp	B1391	infant feces
Bifidobacterium spp	B2529	infant feces
Bifidobacterium spp	B3225	infant feces
Bifidobacterium spp	B7710	pre-term newborn feces
Bifidobacterium spp	B7740	pre-term newborn feces
Bifidobacterium spp	B7840[b]	pre-term newborn feces
Bifidobacterium spp	B7947[c]	pre-term newborn feces
Bifidobacterium spp	B7958[d]	pre-term newborn feces
Bifidobacterium spp	B8452[e]	pre-term newborn feces

[a] strain identified as *B .longum* subsp. *longum* within this work.

[b] strain identified as *B .breve* within this work.

[c] strain identified as *B. breve* within this work.

[d] strain identified as *B.longum* subsp. *longum* within this work.

[e] strain identified as *B. pseudocatenulatum* within this work.

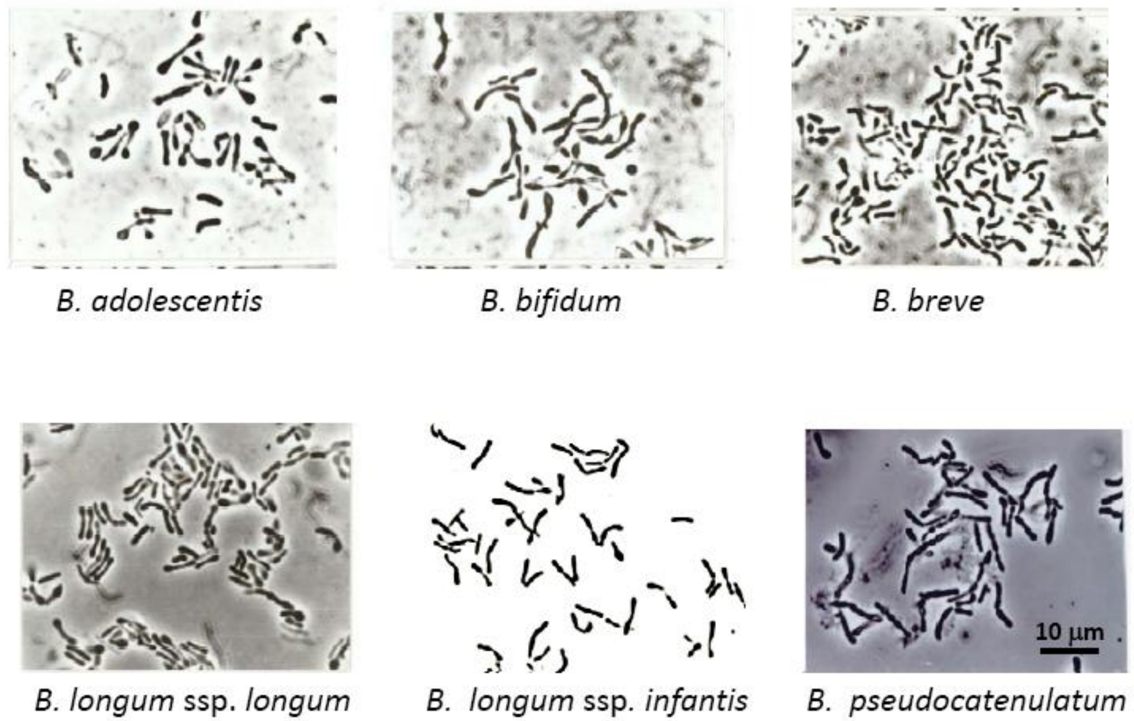


Figure 14 *Bifidobacterium* species predominat in infant microbiota

Table 7 Composition of TPY broth

TPY	g/l
Tryptone	10.0 g
Pepton	5.0 g
Glucose	15.0 g
Yeast extract	2.5 g
K_2HPO_4	1.5 g
$MgCl_2.6H_2O$	0.5 g
Cistein-HCl	0.5 g
Tween 80	0.5 g
pH	6.5

9.2 Antagonistic strains (potentially pathogenic) and culture conditions

The strains used as antagonistic microorganisms were: *E.coli* ATCC 11105*, S. enteriditis* M94 strain and *C. difficile* M216 strain (both isolated from hospitalized patients and available at BuSCoB), *C. jejuni* CIP 70.2^T (from the Collection de l'Institut Pasteur, Paris, France) and two gas-forming coliforms isolated from faeces of colicky infants, *Klebsiella pneumoniae* GC6a strain and *Enterobacter cloacae* GC23a (Savino et al. 2011). The *E. coli*, *S. enteriditis, K. pneumonie* and *E. cloacae* strains were cultivated in Nutrient Broth (NB) (Oxoid, ltd., Basingstoke, Hampshire, England) aerobically at 37°C. *C. difficile* M216 strain was grown in Brain Heart Broth (Merck) and incubated under anaerobic condition at 37°C; *C. jejuni* CIP 70.2^T strain were grown on Nutrient agar (Oxoid, Ltd., Basingstoke, Hampshire, England) containing 5% sheep blood at 42 °C under microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) generated by using the CampyGen Atmosphere Generation System (Oxoid, Ltd., Basingstoke, Hampshire, England) in anaerobic jars for 24-48 hours. Thereafter, one colony of *Campylobacter* was transferred into NB (Nutrient broth) (Oxoid, Ltd., Basingstoke, Hampshire, England) supplemented with 5 % of Laked Horse Blood (Oxoid, Ltd., Basingstoke, Hampshire, England), kept under microaerophilic conditions for 48 hours at 42 °C and then used for the experiment.

The identification of *E.coli* ATTC 11105, *E. clocae* GC6a, *K. pneumoniae* GC23a and *S. enteriditis* M94 were confirmed using BBL Enterotube ™ II (BD, NJ, USA). In **table 8** are reported all the antagonistic strains used in this work and their original habitat.

Table 8 List of the 6 antagonistic strains used in this study and their original habitat

Species	Strain	origin
Escherichia coli	ATCC 11105™	collection strain (unknown origin)
Salmonella enteriditis	M94	hospitalized patient
Clostridium difficile	M216	hospitalized patient
Campylobacter jejuni	CIP 70.2™	bovine feces
Enterobacter cloacae	GC6a	colicky infant feces
Klebsiella pneumoniae	GC 23a	colicky infant feces

9.3 *In vitro* inhibition of antagonistic strains

9.3.1 Agar spot test using living cells

To assess the antimicrobial activity of *Bifidobacterium* spp. strains against selected bacteria (*E.coli* ATCC 11105*, S. enteriditis* M94*, K. pneumoniae* GC23a strain and *E. cloacae* GC 6a were used for all the 46 strains, whereas *C. jejuni* CIP 70.2^{T} and *C. difficile* M216 only for 16 selected strains) the protocol described by Santini et al. (2010) was employed. TPY agar was poured in petri dishes. 10µl of each *Bifidobacterium* o.n. culture, having a absorbance at 600 nm (A600) of approximately 0.7-1, corrisponding to the exponential phase of the growth, were spotted onto appropriate agar plate and, once dried, the plates were incubated in anaerobic conditions for 24-48 hours at 37°C.

Subsequently, the plates were overlaid with 10 ml of nutrient broth 0.7% of agar, containing 100 µl of each antagonistic cell suspension having A600 of 0.1. The petri were incubated for 24 hours at different conditions depending on the antagonistic strain used and the inhibition areas were measured 5 µl of acetic acid (1 M) was used as a positive control and sterile TPY broth at pH 6.5 were used as a negative control.

Each assay was performed in triplicate.

9.3.2 Antimicrobial activity of *Bifidobacterium* spp. culture supernatants

This assay was performed with the 16 strains showing the most interesting antimicrobial activity in the previously described assay and, as a negative control, a *Bifidobacterium* strain not showing any antagonistic activity in the spot agar assay (B7710). Cell-free supernatants were obtained by centrifuging TPY bifidobacteria o.n. cultures (15000 x *g*, 20 min at 4 °C) followed by filtration through a 0.22 µm pore-size cellulose acetate filter. An aliquot of the supernatant was adjusted to pH 7. The antagonist strains used in this assay were: *E.coli* ATCC 11105*, S. enteriditis* M94*, K. pneumoniae* GC23a and *E. cloacae* GC23a. The antagonistic strains were grown in NB until the A_{600} of 0.9 and used to inoculate 96-well plates. Each well contained: 100 µl of double concentrated NB, 25 or 50 µl of *Bifidobacterium* spp. cell-free supernatant (both neutralized and non-neutralized), corresponding to a v/v percentage of 12.5 and 25, respectively, and water to 200 µl of total volume. 1 % v/v inoculum of the antagonistic strain was added.

Posive controls were prepared by using 50 µl of fresh NB without any supernatants. The 96-well plates were incubated aerobically at 37 °C for 22 h; A_{620} was periodically evaluated in a multiwell plate spectrophotomer (Multiskan, Thermo Electron, Oy, Vaanta, Finland).

9.4 Genetic typing of the strains

9.4.1 Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR)

Total DNA was extracted from 10 ml of overnight pure cultures and purified using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). ERIC-PCR patterns of *Bifidobacterium* strains were obtained following the procedure described by Ventura et al. (2003). Primers ERIC-1 (5'ATGTAAGCTCCTGGGGATTCAC-3') and ERIC -2 (5'AAGTAAGTGACTGGGGTGAGCG-3') were used. The 20 µl reaction mixture contained 10 µl of HotStart Taq Plus Master Mix Kit (Qiagen, West Sussex, UK), 1µM of each primer, 1.5mM $MgCl_2$ (Qiagen). PCR reactions were run in a Veriti Thermal Cycler (Applied Biosystem, Foster City, CA, USA). The reference strains used in this study were: *B. pseudocatenulatum* ATCC 27917^T, *B. catenulatum* ATCC 27539^T, *B. breve* ATCC 15700^T, *B. bifidum* DSM 20456^T, *B. longum* subsp. *longum* ATCC 15707^T, and *B. longum* subsp. *infantis* ATCC 15697^T.

9.4.2 PCR with genus-specific and specie-specific primers

Bifidobacterium genus-specific PCR was performed on total DNA using 16S rDNA-targeted primers Bif64-f (GGGTGGTAATGCCGGATG) and Bif662-r (CCACCGTTACACCGGGAA) (Satokari et al. 2001). Species identification was carried out using species-specific PCR primers described by Matsuki et al. (1999).

PCR was carried out in a total volume of 25 µl of reaction mixture containing 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 2.5 mM of $MgCl_2$ (Applied Biosystems, Foster City, Ca), 200 µM each dNTP (Fermentas GmbH**,** St. Leon-Rot, Germany) 25 µM of each primer (see **table 9**) , 0.45 U of Taq DNA polymerase (Fermentas) and 1 µl of template DNA. The PCR amplification program consisted of one cycle of 94 °C for 5 minutes, then 35 cycles of 94 °C for 20 seconds, 55 °C for 20 seconds, and 72 °C for 30 seconds, and finally one cycle of 72 °C for 5 minutes. Amplifications were carried out with a DNA thermocycler ((Biometra, Göttingen, Germany). The amplification products were

then separated by electrophoresis in 1.5% (w/v) agarose gel and ethidium bromide (0.5 g/ml) staining was performed to observe the presence of bands under UV transillumination (Bio-Rad) and photographed. A positive control was performed by using DNA from type strains from our collection and the negative control was performed by using water instead of DNA.

Table 9 Primer sets used for identification of *Bifidobacterium* strains

Microorganism target	Primer	Sequence (5'-3')	Amplicon lengh(bp)
Bifidobacterium adolescentis	BiADO-1 BiADO-2	CTCCAGTTGGATGCATGTC CGAAGGCTTGCTCCCAGT	279
Bifidobacterium bifidum	BiBIF-1 BiBIF-2	CCACATGATCGCATGTGATTG CCGAAGGCTTGCTCCCAA	278
Bifidobacterium breve	BiBRE-1 BiBRE-2	CCGGATGCTCCATCACAC ACAAAGTGCCTTGCTCCCT	288
Bifidobacterium catenulatum group	BiCATg-1 BiCATg-2	CGGATGCTCCGACTCCT CGAAGGCTTGCTCCCGAT	285
Bifidobacterium longum	BiLON-1 BiLON-2	TTCCAGTTGATCGCATGGTC GGGAAGCCGTATCTCTACGA	831
Bifidobacterium infantis	BiINF-1 BiINF-1	TTCCAGTTGATCGCATGGTC GGAAACCCCATCTCTGGGAT	828

9.5 Antibiotic resistance profiles

9.5.1 Minimal inhibitory concentration (MIC)

MIC for 12 antibiotics was determined with the microdiluition assay in TPY broth for the 16 *Bifidobacterium* strains showing the highest antimicrobial activity. 12 antibiotics were selected for this analysis, 8 of which were suggested in the most recent EFSA guidelines (EFSA, 2008), i.e. tetracycline, cefuroxime, kanamycin, chloramphenicol,vancomycin, ampycillin, streptomycin and erythromycin (Sigma-Aldrich, Milan, Italy) whereas other 4 were examined considering their wide use in infant therapy (cefuroxime, amoxicillin, ceftriaxone and clarithromycin) (Sigma-Aldrich). All antibiotic solutions were diluted in distilled water or DMSO or water with Ethanol to prepare stock solution and then additionally diluted with water to final concentration of 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml for the antibiotic resistance

assay. All these dilutions were sterilized by microfiltration with 0,22 μm pore size filter (Millipore, Carrigtwohill, Ireland) before use.

The assay was performed in 96 well plates. In each well we added 20 μl of appropriate dilution of antibiotic, 160 μl of fresh TPY broth and 20 μl of overnight bacterial suspension previously diluted 1:9 in fresh TPY broth to obtain 10^6 CFU/ml. The number of tested bacteria was additionally determined by measuring the optical density (OD) at 620 nm and through the use of a standard McFarland standards. The positive control in assay was a mixture of bacterial suspension (20 μl), broth (160 μl) and the solvent used to prepare antibiotic (20 μl) (with no antibiotic), and the negative control was a mixture of bacterial suspension in water. Two additional controls were included; mixture of water, broth and antibiotic solution and broth only. Growth or inhibition of the strains was determined by measuring the A_{620} at regular time intervals for a total incubation of 24h at 37°C.

Minimal inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic free control well and was measured by reading optical density at 620 nm.

9.5.2 Screening of resistance genes

The presence of known antibiotic resistance genes was determined by PCR reaction using specific primers (see **table 10**): *aph* (3'')-I, *aph* (3'')-II, *aph* (3'')-III coding for kanamycin and neomicine resistance genes (Ouoba et al. 2008), *aad*A, *aad*E, *erm*A coding for streptomycin and erythromycin resistance genes (Ouoba et al. 2008), *tet*(M), *tet*(O), *tet*(W) coding for tetracycline resistance genes (Masco et al. 2006) and blaCTX-M-g1, blaCTX-M-g2, coding for ß-lactam and resistance genes (Van Hoek et al. 2008). The following amplification program was used: 95 °C for 5 min, 35 cycle of 95°C for 1 min, 45-64°C (depending on annealing temperature of each primer), 72°C for 1 min and a final extension step at 72°C for 10 min. *L.casei* L9 was used as positive control for *aph*(3'')-III, *aad*A, *aad*E genes whereas *B. adolescentis* DSM 20087 was used as positive control for *Tet* (W) gene. PCR products were separated by electrophoresis on 1,5% agarose gel.

Table 10 Primer sets evaluated for identification of antibiotic resistence genes

Antibiotic target	Primer	Sequence (5'-3')	References
Kanamycin, neomicine	*aph*(3'')-IF *aph*(3'')-IR	AACGTCTTGCTCGAGGCCGCG GGCAAGATCCTGGTATCGGTCTGCG	Ouoba, 2008
Kanamycin, neomicine	*aph* (3'')-IIF *aph* (3'')-IIR	GCTATTCGGCTATGACTGGGC CCACCATGATATTCGGCAAGC	Ouoba, 2008
Kanamycin, neomicine	*aph* (3'')-IIIF *aph* (3'')-IIIR	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA	Ouoba, 2008
Streptomycin	*aad*A-F *aad*A-R	ATCCTTCGGCGCGATTTTG GCAGCGCAATGACATTCTTG	Ouoba, 2008
Streptomycin	*aad*E-F *aad*E-R	ATGGAATTATTCCCACCTGA TCAAAACCCCTATTAAAGCC	Ouoba, 2008
Erythromycin	*erm*A-F *erm*A-R	AAGCGGTAAAACCCCTCTGAG TCAAAGCCTGTCGGAATTGG	Ouoba, 2008
Tetracycline	*tet*(M)-F *tet*(M)-R	GTTAAATAGTGTTCTTGG AG CTAAGATATGGCTCTAACAA	Masco, 2006
Tetracycline	*tet*(O)-F *tet*(O)-R	GATGGCATACAGGCACAGAC CAATATCACCAGAGCAGGCT	Masco, 2006
Tetracycline	*tet*(W)-F *tet*(W)-R	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	Masco, 2006
ß-lactam	blaCTX-M-g1F blaCTX-M-g1R	GTACAGCAAAAACTTGCCG CTTTCACTTTTCTTCAGC	Van hoek, 2008
ß-lactam	blaCTX-M-g2F blaCTX-M-g2R	CGCTGCATGCGCAGGCG GCAAAACGTTCATCGGCACG	Van hoek, 2008

9.5.3 Plasmid detection

Pure Yield Plasmid Miniprep System kit (Promega) was used to extract and purified plasmid DNA from the 16 *Bifidobacterium* strains showing the highest antimicrobial activity. *B. longum* B2399, which was known to possess two plasmids (Sgorbati et al.1982), was used as positive control for plasmid DNA extraction. Plasmids were separated after electrophoresis on a 0.7% agarose gel during 3.5 h at 100V and visualized in ethidium bromide staining.

9.5.4 Evaluation of the transferability of the antibiotic resistance traits

4 *Bifidobacterium* strains (B632, B1975, B2274, B7840) were used as donor strains, whereas *Bifidobacterium animalis* ATCC 27536, *B. longum* subsp. *suis* PCD733B (Santini et al. 2010), 3 *Bifidobacterium* strains from this study (B1412, B7840, B632), *Lactobacillus plantarum* PCS22 (Nissen et al., 2009), and *Enterococcus faecium* PCD71 (Santini et al., 2010) were used as recipient strains. *Bifidobacterium* strains were

grown overnight as already described, whereas lactic acid bacteria were grown in MRS (DeMan-Rogosa Sharpe) broth (Merck, Darmstadt, Germany) at 37°C in anaerobic or aerobic conditions.

The transferability of the antibiotic resistance traits was assayed following the protocols of Lampkowska et al. (2008) and Ouoba et al. (2008). Donor and recipient strains were cultivated separately to mid exponential growth phase in liquid medium with appropriate antibiotics, and then mixed in 1:1 ratio in a final volume of 200 µl. The mixture was inoculated into 10 ml of TPY broth (which permits the growth of both donor and recipient strains) anaerobically for 24 h at 37 °C. At the end of incubation time, cells were harvested by centrifugation (10 min at 6,000 rpm), resuspended in 1 ml of PBS and plated on donor- and recipient-selective agar plates and on selection plates, i.e. plates in which only the growth of recipient strains having acquired the antibiotic resistance can occur. The same selection plates were also used to estimate the frequency of spontaneous mutations in the recipient strain. To counter select lactic acid bacteria having acquired antibiotic resistance from bifidobacteria, the selection plates were incubated in aerobic conditions. A scheme of the experiments, including the conditions for the selection of the recipients strains, is presented in **table 11**.

Table 11 Evaluation of the transferability of the antibiotic resistance traits from *B. breve* B632, B2274 and B7840 and *B. longum* B1975 to selected recipient strains

Donor strain	Antibiotic resistance assayed*	Recipient strain(s)	Selection plates
B632	Ampicillin (blaCTX-M-g1)	ATCC 27536	TPY+ AMP + TET
	Ampicillin	PCS22	MRS + AMP + aerobiosis**
	Kanamycin	B1412	TPY+ KAN + AMO
	Streptomycin	B7840	TPY+ STR + TET
B1975	Ampicillin (blaCTX-M-g1)	ATCC 27536	TPY+ AMP + TET
	Ampicillin	PCS22	MRS + AMP + aerobiosis
	Kanamycin (aph (3'')III)	B1412	TPY+ KAN + STR
	Amoxicillin (blaCTX-M-g1)	PCD71	MRS + AMP + aerobiosis
B2274	Ampicillin	PCS22	MRS + AMP + aerobiosis
	Tetracycline (tetW)	PCD71	MRS + TET + aerobiosis
	Kanamycin	B1412	TPY + KAN + TRIM
	Streptomycin	B7840	TPY+ STR + KAN
	Amoxicillin	B632	TPY+ AMO + TRIM
	Amoxicillin	PCD71	MRS + AMO + aerobiosis
B7840	Ampicillin (blaCTX-M-g1)	PCD733B	TPY+ AMP + STR
	Ampicillin	PCS22	MRS + AMP + aerobiosis
	Tetracycline (tetW)	B632	TPY+ TET + STR
	Tetracycline	PCD71	MRS + TET + aerobiosis
	Kanamycin	B1412	TPY+ KAN + STR
	Amoxicillin (blaCTX-M-g1)	B632	TPY+ AMO + STR
	Amoxicillin	PCD71	MRS + AMO + aerobiosis

* the resistance genes indicated in brackets has been identified by PCR
** plates were incubated in aerobic conditions to allow the growth only of lactic acid bacteria

AMO = amoxicillin, AMP = ampicillin, CEFT = ceftriaxone, CEFU = cefuroxime, CHL = chloramphenicol, CLA = clarithromycin, ERY = erythromycin, KAN = kanamycin, GEN = gentamycin, STR = streptomycin, TET = tetracyclin, TRIM = Trimethoprim, VAN = vancomycin,

9.6 *In vitro* interaction between *Bifidobacterium* strains and human cells

9.6.1 Growth and maintenance of cell line

The following cell lines were used: small intestinal human epithelial cell line H4 (**Figure 15A**), derived from human foetal tissue and supplied by Massachusetts General Hospital (Prof. W.A. Walker) , and human blood monocytes/macrophages, referred to as TLT (**Figure 15B**) cell line, established in the laboratory of Prof. A. Cencič, Maribor, Slovenia (Cencič and Langerholc, 2010). Cell were routinely grown in Dulbecco Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2mmol/L), penicillin (100 U/ml) and streptomycin (1 mg/ml) . Cells were cultured in flasks or Petri dishes in an incubator with 5% CO_2 at 37 °C. To perform biological assays the cells were seeded in 96 well plates at the concentration of 1×10^6 viable cells/ml, as determined by 0.1% trypan blue viability staining, and incubated for 24 h at 37°C in humidified atmosphere of 5% CO_2. The assays described below were performed with the 16 *Bifibobacterium* strains showing the highest antagonistic activity against the bacteria assayed; they were grown in TPY, harvested by centrifugation at 2000 *g* for 10 min and suspended in DMEM to final concentrations of 1×10^8 CFU/ml. When cell monolayers in 96-well plates were obtained, the strains of *Bifidobacterium* were inoculated in each well at the concentration of 10^7 CFU/ml. In most of the assays described the well known probiotic strain *Lactobacillus rhamnosus* GG (LGG) was used to compare the results obtained. All reagents used for these assays were purchased from Sigma-Aldrich.

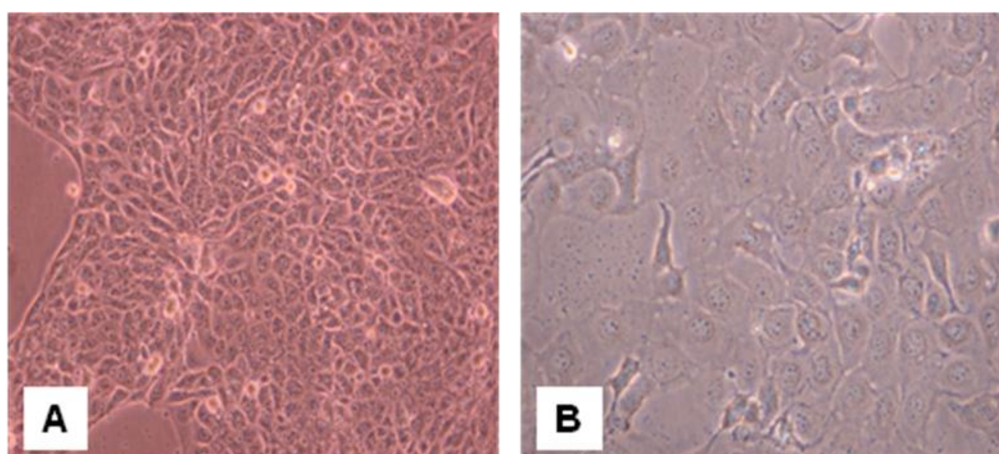


Figure 15 H4 (A) and TLT (B) human cell lines (image kindly provided by Department of Microbiology, Biochemistry and Biotechnology, Faculty of Medicine, University of Maribor, Slovenia)

9.6.2 Cytotoxicity assays

Cytoxicity activity of *Bifidobacterium* spp. strains on cell monolayer of H4 and TLT cell lines was assayed. *Bifidobacterium* strains were inoculated in the wells at the concentration of 10^7 CFU/ml and plates were then incubated under anaerobic conditions at 37°C for 90 minutes, after which free bacteria were eliminated by washing the cell layers three time with phosphate-buffered saline (PBS). 100 µl of DMEM without phenol red and antibiotics and supplemented only with L-glutamine was added to each well, and plates were incubated for 24 hours. Cell viability was measured with crystal violet staining, measuring absorbance at 595 nm (A_{595}), and the value obtained was compared to the A_{595} obtained in non treated cells (i.e. cells not exposed to probiotics).

9.6.3 Adhesion assay

The capability of selected *Bifidobacterium* strains of adhering to H4 and TLT cell lines was assayed. The cell monolayers were washed with PBS and probiotic strains were applied to the wells to have a concentration of 9.4 LOG(CFU/sqm). Plates were incubated for 90 minutes at 37 °C. Subsequently, the monolayers were washed three times with PBS, then cells with adherent bacteria were harvested with trypsin and the number of bacteria adhering to the cell lines was counted. Results of attached bacteria cells were expressed as % of adherent bacterial cells compared to initial inoculum.

9.6.4 Mitochondrial activity assay

The metabolic activity of H4 and TLT cell lines after exposure to *Bifidobacterium* strains was measured by evaluation of their mitochondrial function as index of cell viability (Bergamini et al.,1992; Ivec et al., 2007). Bacterial pellet was resuspended in DMEM without phenol red and supplements. After 90 minutes of bacterial exposure to cell monolayers, cells were washed and reincubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM was added to each well and incubated for 75 minutes. Solubilization of MTT reduction product (i.e. formazan) was achieved by addiction of 0.04% HCl in isopropanol; solubilized formazan was evaluated at $A_{650.}$ Results are expressed as: (A_{650} of treated wells - A_{650} of untreated wells)/ A_{650} of untreated wells × 100.

9.6.5 Determination of Reactive Oxygen species (ROS): NO, H_2O_2

To measure the amount of NO and H_2O_2 released by H4 and TLT cells in the presence of probiotics, bacterial pellets were resuspended in DMEM supplemented with only L-glutamine and added to confluent monolayers of H4 and TLT cells. After 90 minutes of bacterial treatment, monolayers were washed and reincubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2. The NO concentration was determinated by measuring the accumulation of nitrate using a modified Griess reagent (Sigma), according to the Griess reaction (Green et al., 1982, Ivec et al. 2007; Pipenbaher et al., 2009). The release of H_2O_2 was determined by transferring 50 µl of supernatant into a 96-well plate and adding 50 µl of 0.01% peroxidase and 100 µl of tetramethylbenzidine (TMB) solution (diluted in water 1:1). Absorbance was measured at 450 nm after 15 minutes of incubation at room temperature. Constitutive H_2O_2 production by bifidobacteria was evaluated by incubating bifidobacteria in DMEM; the amount of H_2O_2 produced by bifidobacteria was subtracted from the amount produced by the cells.

9.6.6 Dot-blot for interleukin 6

Interleukin-6 (IL-6) in supernatants of H4 and TLT cells after probiotc treatment was detected using the dot-blot technique as described by Ivec et al. (2007). Supernatants were blotted onto nitrocellulose membrane (Pierce, Rockford, USA) under gravity with a Bio-Rad Dot Blot apparatus (Bio-Rad Laboratoires, Hercules, USA). Membrane was incubated with the primary antibody, a rabbit anti-human IL-6 (Sigma) and with a secondary antibody (an anti IgG horseradish peroxidise-conjugated antibody). Proteins were visualised with the supersignal West Pico chemioluminescent substrate system (Pierce) and BiomaxMR-1 film (Sigma Kodak, USA). Supernatants of monolayers not treated with bacteria were used as negative control, whereas *L. casei* Shirota and LGG were used as positive controls. To avoid false positive results, all samples were evenly tested against the sole secondary antibody.

9.7 Experimental design, statistical analysis and strain selection criteria

For the different trials the adopted experimental scheme was a fully randomised design. All the tests were performed in triplicate. Data on spot agar tests, cytotoxicity assay, adhesion test, mitochondrial activity test and ROS (NO, H_2O_2) production were subjected to one way analysis of variance (ANOVA) by using the GLM procedure of the SAS statistical package. Means were subjected to Fisher's test (SAS, 1988). When treatments were significant according to Fisher's test, corresponding means were differentiated by the SNK multiple range test at the 0.05 level of probability.

The correspondence analysis (CA) was applied to the fingerprinting pattern obtained from ERIC-PCR of *Bifidobacterium* reference strains and investigated strains. CA is a statistical method for visualising the association between levels of a two-way contingency table (Benzecri 1992). Banding profiles were scored as presence/absence of individual fragments in each investigated strain. The contingency table was analyzed by CA module of Statistica Software (ver. 7.1, StatSoft, Tulsa, Oklahoma, USA). Plotting the first two dimensions of the coordinates of cases (ERIC-PCR bands) and variables (strains) gave a global view of the correspondence among reference and investigated strains, and band patterns. The first and second dimensions explained 34 and 28% of the total variability, respectively.

A first strain selection was based on antimicrobial activity against *E.coli, S. enteriditis, K. pneumoniae* and *E. cloacae* allowing the choice of the 16 best performing strains out of the original 46 strains. Among the 16 strains, four bifidobacteria were selected on the basis of a synthetic index, calculated as follows: the outputs of different analyses (spot agar tests, antibiotic resistance or sensitivity assay, cytotoxicity test, adhesion assay, mitochondrial dehydrogenase activity, NO and H_2O_2 production) were transformed into relative percentages by giving the 100 value to the strain showing the best performance in each test. A correction factor of 0.5 was given to the mitochondrial dehydrogenase activity, NO and H_2O_2 production tests, in order to give more importance to the other parameters which are defined in the EFSA guidelines (EFSA, 2005). IL-6 production was not considered in this evaluation as it is not a quantitative test. These procedures allowed to select 4 strains which were checked for the transferability of the antibiotic resistance traits to other gut bacteria and were then deposited to the DSMZ culture collection.

Chapter 10. Evaluation of the most effective prebiotic fiber

10.1 Prebiotic activity assay

The ability of the 4 selected *Bifidobacterium* strains (B632, B1975, B2274, B7840) to use as the sole carbon source and energy source different polisaccharide fibers was investigated (**Table 12**).

The assay was performed by adding 2% (v/v) of an overnight-incubated culture (A_{620} 0.7) of each *Bifidobacterium* strain to separate tubes containing a modified TPY broth (i.e. containing half the concentration of tryptone, peptone and yeast extract) supplemented with 1% (w/v) glucose or 1% (w/v) prebiotic fiber as the sole carbon source. To confirm that negligible growth occurred from use of indigenous carbon sources present in the base medium, strains were also grown on base medium with no added carbon source. The assay was performed in 96 well plates, and the bacterial growth was determined by measuring the A620 nm after 0, 6, 24, 30 and 48 h of incubation at 37°C in anaerobic atmosphere. In addition, overnight cultures of coliforms of gut origin, i.e. *E. coli* ATCC25645, *K. pneumoniae* GC 23a and *E. cloacae* GC 6a were mixed in a 1:1:1 ratio (A_{620} 0.1) (referred to as enteric mixture), then added at 2% (v/v) to separate tubes containing M9 medium (Eisenstadt et al., 1994) with 1% (w/v) glucose or 1% (w/v) prebiotic fiber. The cultures were incubated at 37°C under anaerobic conditions, and the bacterial growth was determined by measuring A620 nm at the same incubation time and incubation conditions of bifidobacteria. Each assay was replicated three times.

The growth curves for *Bifidobacterium* strains, for each enteric microrganism and for enteric mix grown in the presence of tested prebiotic fibers were generated by plotting the mean number of A620 versus incubation time (0, 6, 24 and 48 h).

The prebiotic activity score was determined by a modification of the formula described in Huebner et al. (2007) as follows:

= {(A620 nm of probiotics strain on the prebiotic at 24 h – A620 nm of probiotics strain on the prebiotic at 0 h)/ A620 nm of probiotics strain on glucose at 24 h – A620 nm of probiotics strain on the glucose at 0 h)} – {(A620 nm of enteric mixture on the prebiotic at 24 h – A620 nm of enteric mixture on the prebiotic at 0 h)/ (A620 nm enteric mixture on glucose at 24 h – A620 nm of enteric mixture on the glucose at 0 h)}

Table 12 List of prebiotic fibers used to evaluate the capability of stimulating bifidobacteria growth.

Carbohydrate type	Composition and DP (where available)	Acronim	Commercial name	Provider
Fructooligosaccharide	f-nistose 11.3%, nistose 42.5%, l-ketose 43.1%, saccarose 2.4 % DP 2 to 5	**Actilight**	Actilight 950P[1]	Beghin-Meiji, Francia
Fructooligosaccharide	DP < 8	**FOS**	FOS	Probiotical SpA Novara, Italy
Inulin	DP 9 to 12	**Frutafit**	Frutafit[2]	Sensus, Netherlands
Inulin	inulin 86% sugars 14 % DP < 10	**Beneo**	Beneo HSI[3]	Orafti, Belgium
oligofructose enriched inulin (patented blend of inulin and oligofructose)	oligofructose 92% sugars 8 % DP N/A	**Synergy**	Raftilose Synergy 1[3]	Orafti, Belgium
Inulin	inulin 100% DP > 23	**Raftiline**	Raftiline HP[3]	Orafti, Belgium
Galactooligosaccharide	GOS 59% Lactose 21% Glucose 19% Galactose 1%	**Vivinal**	Vivinal GOS[4]	Borculodomo, Netherlands
Galactooligosaccharide	GOS DP N/A	**CUP-Oligo**	CUP-Oligo[5]	Azelis SpA, Milano, Italy
α-glucooligosaccharide	DP>3	**BioEcolians**	BioEcolians[6]	Solabia group, Pantin Cedex, France
Arabinogalactan (*Larix occidentalis* fiber)	Arabinogalactan	**Larch fiber**	Arabinex [7]	Thorne research, Dover, USA
Partially hydrolysed guar gum (PHGG)	PHGG	**Benefibra**	Benefibra[8]	Novartis Pharma Spa, Origgio (Va), Italy

More information about the products are available online at the following websites:
[1] www.beghin-meiji.com/actilight
[2] www.sensus.us
[3] www.orafti.com
[4] www.vivinalgos.com
[5] www.kowa-europe.com/food/
[6] www.solabia.fr/Solabia/SolabiaNutrition.nsf/
[7] thorne.com/Products
[8] www.benefibra.it

Chapter 11. Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn microbiota

11.1 Newborn study design and sample collection

From October 2011 to January 2012, a study with 31 newborns was carried out aimed at evaluating the effect on the gut microbiota after antibiotic administration to their mother every 4 hours before the delivery. 14 infants were born by mothers resulted negative to Group B *Streptococcus* (GBS) and 17 infants by mothers, positive to GBS and treated with 2g of ampicillin.

All the subjects were recruited from the Neonatal Intensive Care Unit of the University of Bologna (Sant'Orsola Hospital) led by Dr. Luigi Corvaglia. Further inclusion criteria were: infants aged between 6-7 days, with a regular birth weight. Only infants born by natural delivery and breastfed were enrolled in order to reduce variability in the intestinal microbiota consequent to diet and delivery (Penders et al., 2006).

About 200 g faeces were collected for each subject. Each sample was stored at 80°C, immediately after collection, in a numbered screw-capped plastic container, until they were processed for DNA extraction.

11.2 DNA extraction from faecal samples

DNA extraction from faecal samples was performed with QIAamp DNA Stool Mini Kit [Qiagen, Cat. No. 51504]. DNA purity and concentration was evaluated with a spectrophotometer (Beckman coulter, DU®730). Extracted DNA was stored at -20°C.

11.3 Real-Time PCR assays

The assays were performed with a 20 µl PCR amplification mixture containing 10 µl of Fast SYBR® Green Master Mix (Applied Biosystems), optimized concentrations of primers (**table 13a-b**), H_2O molecular grade and 2 µl DNA extracted from faecal samples at a concentration of 2.5 ng/µl for all the assay except *C. difficile* quantification. For *C. difficile* quantification DNA extracted from faecal samples was not diluted. The primer concentrations were optimized through primer optimization matrices in a 48-well plate and evaluating the best Ct/ΔRn ratio. The data obtained are

then transformed to obtain the number of bacterial Log cells/g faeces according with the rRNA copy number available at the rRNA copy number database (**Table 13c**) (Klappenbach *et al.*, 2001; Lee *et al.*, 2009). Equations and coefficients of determination for the different assays are reported in **table 13d**.

Data of microbial counts were subjected to one-way analisys of variance in order to evidence significant differences between treated and control group odf newborns.

Table 13a Primer sets used for identification of *Bifidobacterium* strains

Microorganism target	Primer	Sequence (5'-3')	Amplicon lengh(bp)	Refences
Escherichia coli	Eco-F Eco-R	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	340	Malinen, 2003
Clostridium difficile	Cdiff-F Cdiff-R	TTGAGCGATTTACTTCGGTAAAGA TGTACTGGCTCACCTTTGATATTCA	114	Penders, 2006
Bifidobacterium spp.	BiTOT-F BiTOT-R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	243	Rinttilä, 2004
Lactobacillus spp	Lac-F Lac-R	GCAGCAGTAGGGAATCTTCCA GCATTYCACCGCTACACATG	349	Castillo, 2006
Bacteroides fragilis group	Bfra-F Bfra-R	CGGAGGATCCGAGCGTTA CCGCAAACTTTCACAACTGACTTA	92	Penders, 2006

Table 13b Cycles and primers concentration for qPCR using SybrGreen chemistry

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

Table 13c 16S rDNA copy number of different genera and species

Genus- Species Targets	Primer Targets	Gene copy number mean*
E.coli	16S rDNA	7
C.difficile	16S rDNA	9,5
Bifidobacterium spp.	16S rDNA	3,57
Lactobacillus spp.	16S rDNA	5,71
B.fragilis group	16S rDNA	6

* (Klappenbach et al., 2001; Lee et al., 2009)

Table 13d qPCR equations and R^2 for the different assay

Target	Equation	R^2
Lactobacillus spp.	Ct= -3.666x + 39.31	0,998
Bifidobacterium spp.	Ct= -3.579x + 39.615	0.998
Bacteroides fragilis group.	Ct= -3.925x + 47.69	0.995
Escherichia coli	Ct= -3.617x + 44.434	0.999
Clostridium difficile	Ct= -3.386x + 38.556	0.989

PART 4: RESULTS

Chapter 12. Selection and characterization of Bifidobacterium strains

12.1 Antimicrobial activity with the spot agar test

The antimicrobial activity with the spot agar test was evaluated measuring the *radius* of the target strain's inhibition halo that surrounds the *Bifidobacterium* spot. The results obtained with the 46 *Bifidobacterium* strains against *E. coli, E. cloacae, K. pneumoniae* and *S. enteriditis* evidenced antimicrobial activity to varying degrees (**Table 14**). An example of the halos obtained is shown in **Figure 16,** three strains (B2531, Re11, B7710) did not show any inhibition halo against all the indicator strains, 27 strains showed inhibition halo's *radius* not higher than 0.5 cm, whereas 14 strains (Re12, B632, B1412, B1975, B2021, B2055, B2091, B2101, B2150, B2192, B2195, B2274, B7840, B7958) showed inhibition halo's *radius* lower than 0.5 cm against all strains, including the two gas-forming coliforms isolated from colicky infants. The elaboration of the results with the ANOVA test allowed to indicate these 14 strains as the most performing; however, we decided to include two more strains (B7947 and B8452) for further studies considering their hight anti-microbial activity against *E. coli*, which is the most abundant coliform in the infant gut, and their potential interest as pre-term isolated strains.

These 16 strains were then assayed against *C. jejuni* and *C. difficile* as antagonistic microorganisms. The results obtained (**Table 15**) evidenced that all *Bifidobacterium* strains except for B2101 were capable of inhibiting both antagonistic microorganisms. Among them, 8 strains (B632, B1412, B1975, B2055, B2192, B2274, B7840, B8452) showed a marked activity against the two pathogens.

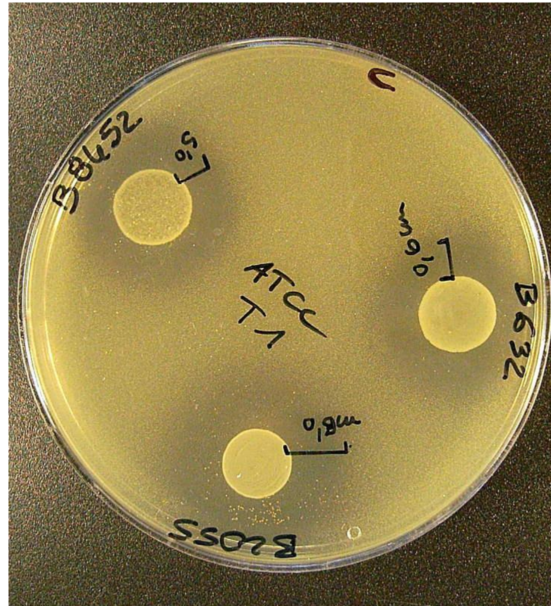


Figure 16 Spot agar test of 3 *Bifidobacterium* strains (B632, B2055, B8452) against *E. Coli* (ATCC 11105™).

Table 14 Evaluation of antimicrobial activity of 46 Bifidobacterium strains against 4 antagonistic strains (*E. coli, E. cloacae, K. pneumoniae* and *S. enetriditis*) expressed as average *radius* (in cm) of the inhibition halos obtained on TPY plates in the agar spot test. The average of the values obtained for each *Bifidobacterium* strain is presented in the right column; mean values followed by different letters (in brackets) are statistically different at $P<0.001$.

Strain	Antimicrobial activity				
	E. coli ATCC 11105	*E. cloacae* GC 6a	*K. pneumoniae* GC 23a	*S. enteriditis M 94*	Average inhibition *radius* (cm)
B1968	0.2	0.3	0.2	0.2	0.22(gh)
B2009	0.4	0.4	0.3	0.4	0.37(eh)
B2531	0	0	0	0	0 (h)
B2091	0.6	0.6	0.6	0.7	0.62(bg)
B2274	0.8	1	1	1.3	1.02(ab)
B2021	0.6	0.9	0.9	1	0.85(ae)
B632	1.2	0.8	0.9	1.2	1.02(ab)
B1501	0.5	0.1	0.2	0.1	0.22(gh)
B2150	0.6	1	0.8	1	0.85(ae)
B2142	0.4	0.4	0.5	0.5	0.45(dh)
B2228	0.2	0.3	0.1	0.2	0.20(gh)
B626	0.1	0.3	0.1	0.3	0.20(gh)
B633	0.2	0.2	0.1	0	0.12(gh)
B2136	0.4	0.3	0.3	0.6	0.40(dh)
B2023	0.7	0.2	0.2	0.7	0.45(dh)
B2195	0.5	0.9	0.7	1.1	0.80(af)
B2210	0.2	0.3	0.1	0	0.15(gh)
B1412	1.2	1.3	0.9	1	1.10(a)
B651	0.1	0.2	0.1	0.3	0.17(gh)
B1915	0.1	0.2	0.1	0.3	0.17(gh)
B1860	1.1	0.1	0.3	0	0.37(eh)

Strain	**Antimicrobial activity**				
	E. coli ATCC 11105	*E. cloacae* GC 6a	*K. pneumoniae* GC 23a	*S. enteriditis M 94*	Average inhibition *radius* (cm)
Re 6	0.7	0	0	0	0.17(gh)
B1629	0.2	0.5	0.5	0.4	0.40(dh)
Re11	0	0	0	0	0 (h)
Re12	0.9	0.8	0.8	1	0.87(ad)
B2101	0.9	0.9	1	1	0.95(ac)
B197	0.9	0.7	0.6	1.2	0.85(ae)
B1482	0.5	0	0.4	0	0.22(gh)
B2327	0.3	0.3	0	0.6	0.30(gh)
B2212	0.5	0	0.1	0	0.15(gh)
B2192	0.9	1	0.7	1.5	1.02(ab)
B2055	0.7	0.5	0.5	0.5	0.55(cg)
B1993	0.1	0.3	0.2	0	0.15(gh)
B1996	0.5	0.6	0.4	0.2	0.42(dh)
B7311	0.3	0.4	0.5	0.7	0.47(dh)
B7162	0.3	0	0	0.3	0.15(gh)
B1279	0.5	0.5	0.4	0.4	0.45(dh)
B1391	0.1	0.1	0.3	0.2	0.17(gh)
B2529	0	0.1	0.1	0.3	0.12(gh)
B3225	0.5	0.3	0.2	0.3	0.32(fh)
B7710	0	0	0	0	0 (h)
B7740	0	0.5	1	0.5	0.50(dh)
B7840	0.7	1	0.6	1	0.82(ae)
B7947	0.7	0.4	0.3	0.5	0.47(dh)
B7958	0.7	0.6	0.8	1.1	0.80(af)
B8452	0.6	0.1	0.6	0.2	0.37(eh)

Table 15 Antagonistic activity of 16 selected *Bifidobacterium* strains against *C. jejuni* LMG8841 and *C. difficile* M216 expressed as average *radius* (in cm) of the inhibition halos obtained on TPY plates in the agar spot test ; mean values followed by different letters (in brackets) are statistically different at $P<0.05$ for *C.jejuni* assay and $P<0.01$ for *C. difficile*.

Strain	***C. jejuni* LMG8841**	***C. difficile* M216**
Re 12	1.1(a)	0.4(ab)
B 632	0.8(ab)	0.7(a)
B1412	1.1(a)	0.8(a)
B1975	0.8(ab)	0.7(a)
B2021	1.0(ab)	0.4(ab)
B2055	1.0(ab)	0.6(a)
B2091	0.8(ab)	0.4(ab)
B2101	0.8(ab)	0.0(b)
B2150	0.8(ab)	0.4(ab)
B2192	1.0(ab)	0.6(a)
B2195	1.2(a)	0.5(a)
B2274	1.0(ab)	0.7(a)
B7840	1.4(a)	0.6(a)
B7947	0.3(b)	0.3(ab)
B7958	1.1(a)	0.4(ab)
B8452	0.8(ab)	0.7(a)
P	0.05	0.01
LSD	0.4	0.3

12.2 Antimicrobiobial activity of *Bifidobacterium* culture supernatants against coliforms and *S. enteriditis*

In order to better characterize the antagonistic activity of bififdobacteria, the capability of crude and neutralized supernatants of inhibiting the growth of *E.coli* ATCC 11105*, S. enteriditis* M94*, K. pneumoniae* GC23a and *E. cloacae* GC6a was assayed. The neutralized supernatant was referred to as NCS whereas the non-neutralized one was referred to as CS. The culture supernatants, CS and NCS, of the 16 *Bifidobacterium* strains showing the highest antimicrobial activity (listed in **Table 14**), plus one strain (B7710) as negative control, were used for evaluating the inhibiting activity towards the selected target strains. The majority of *Bifidobacterium* supernatants were capable of exerting their inhibiting activity mainly when non-neutralized, whereas the inhibitory activity of 4 strains (B632, B1975, B2274 and B7840) was evidenced both with CSs and NCSs. **Figure 17** shows details of the experiments performed with B632: the inhibitory activity of B632 towards *E. coli* and *S. enteriditis* was clearly evident in the early hours of incubation (**Figure 17A-B**) with no differences in the use of CS and NCS, whereas the inhibitory activity towards *E. cloacae* and *K. pneumoniae* was less marked with respect to the other target strains (**Figure 17C-D**) and, moreover, it was more evident when the non-neutralized supernatants was used. The profiles obtained with B1975 showed a greater inhibition when the supernatants were used against *E. coli* and *S. enteriditis* (**Figure 18A-B**) and generally, there were no differences by using CS and NCS. Regarding the profiles of B2274 (**Figure 19**) CS showed an almost total inhibition of the growth of *E. cloacae* and *K. pneumoniae* (**Figure 19 C-D**) at the highest concetration assayed. The inhibitory activity of B7840 is more marked during the first few hours of incubation and less evident as the incubation proceeded (**Figure 20**). No inhibitory activity against all the antagonistic strains was evidenced by the B7710 strain (data not shown).

In order to further characterize the inhibitory activity of B632, NCS was concentrated 10 times by liophylization and the experiment was repeated. No particular differences were observed compared to the previous results. Threfore, althought the interpretation of these results does not clarifly the nature of the inhibitory activity, the presence of a proteinaceus molecules as inhibitory factor cannot be excluded.

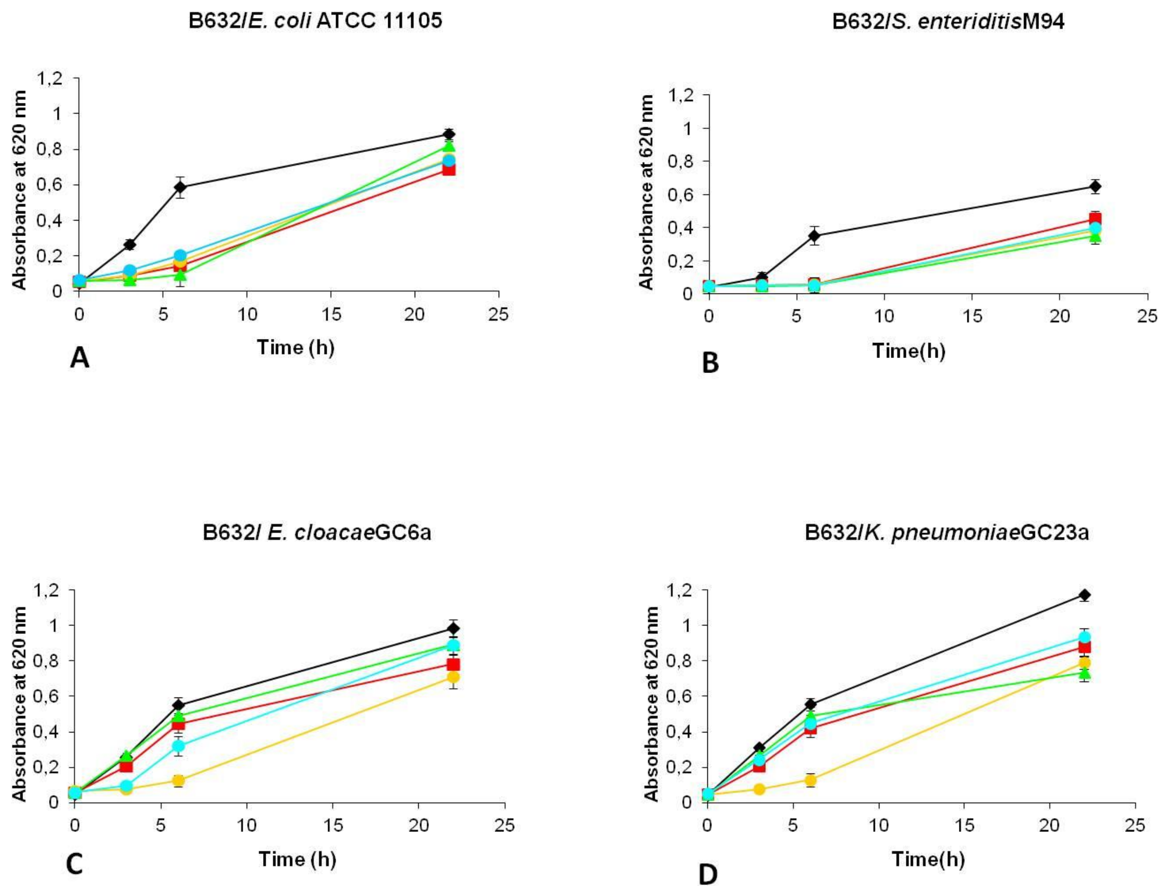


Figure 17 Effect of culture supernatants (CS) and neutralized culture supernatants (NCS) of of *B. breve* B632 on the growth of *E. coli* ATCC 11105 (A), *S. enteriditis* M94 (B), *E. cloacae* GC6a (C), *K. pneumoniae* GC23a (D), control with 50 µl NB (black), 25 µl CS (red), 50 µl CS (yellow) , 25 µl NCS (green), 50 µl NCS (light blue).

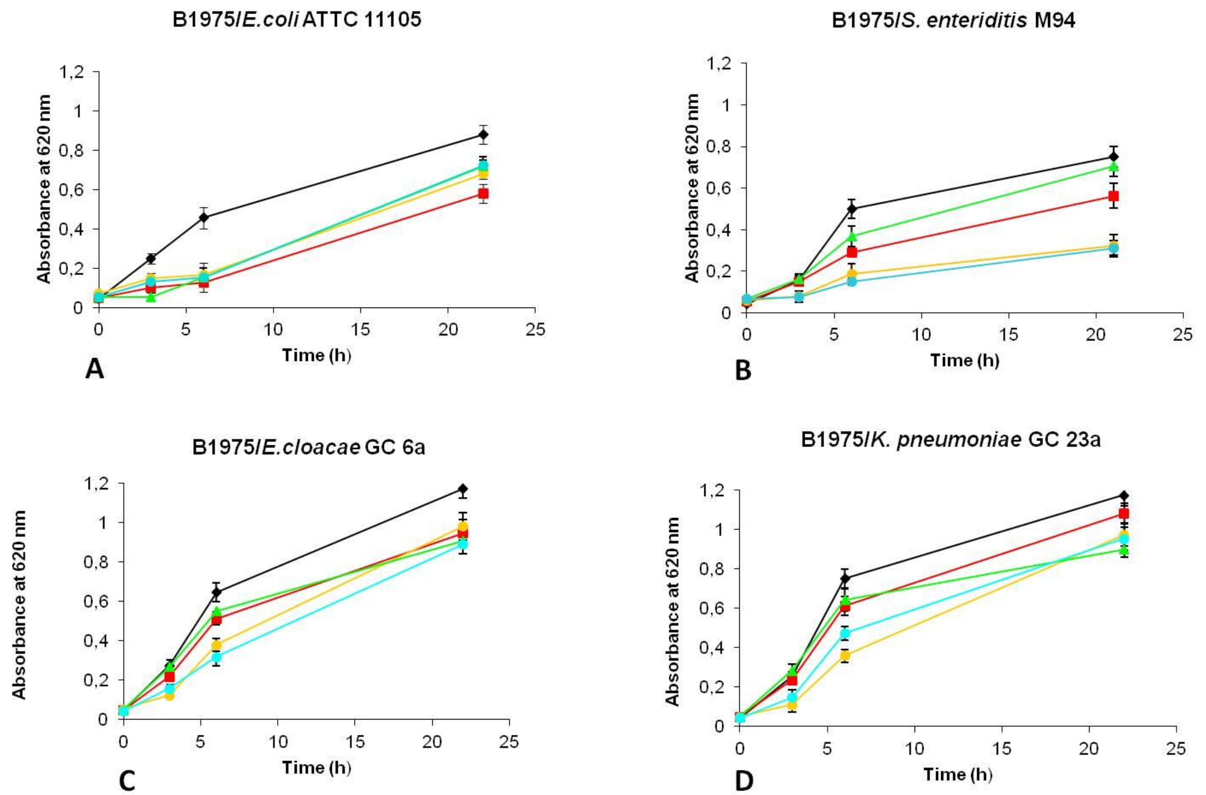


Figure 18 Effect of culture supernatants (CS) and neutralized culture supernatants (NCS) of of *B. longum* B1975 on the growth of *E. coli* ATCC 11105 (A), *S. enteriditis* M94 (B), *E. cloacae* GC6a (C), *K. pneumoniae* GC23a (D), control with 50 µl NB (black), 25 µl CS (red), 50 µl CS (yellow) , 25 µl NCS (green), 50 µl NCS (light blue).

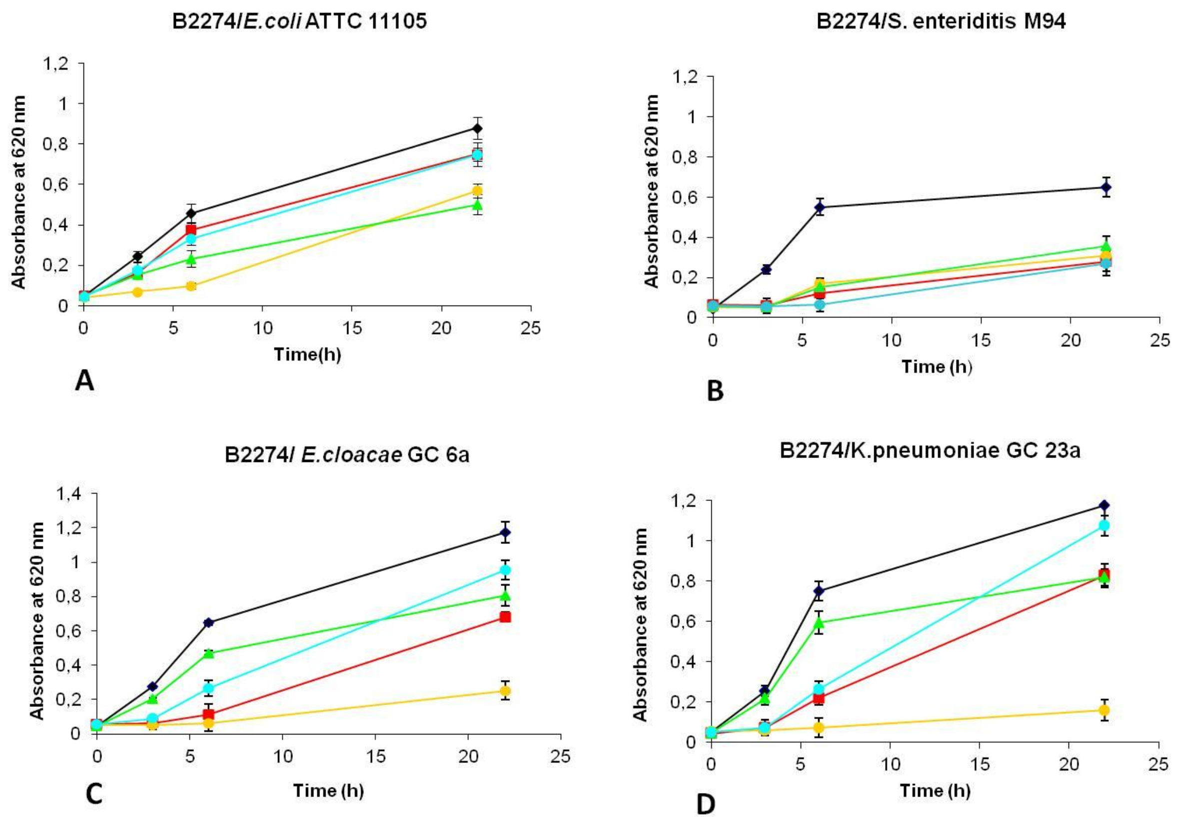


Figure 19 Effect of culture supernatants (CS) and neutralized culture supernatants (NCS) of of *B. breve* B2274 on the growth of *E. coli* ATCC 11105 (A), *S. enteriditis* M94 (B), *E. cloacae* GC6a (C), *K. pneumoniae* GC23a (D), control with 50 µl NB (black), 25 µl CS (red), 50 µl CS (yellow) , Δ 25 µl NCS (green), 50 µl NCS (light blue).

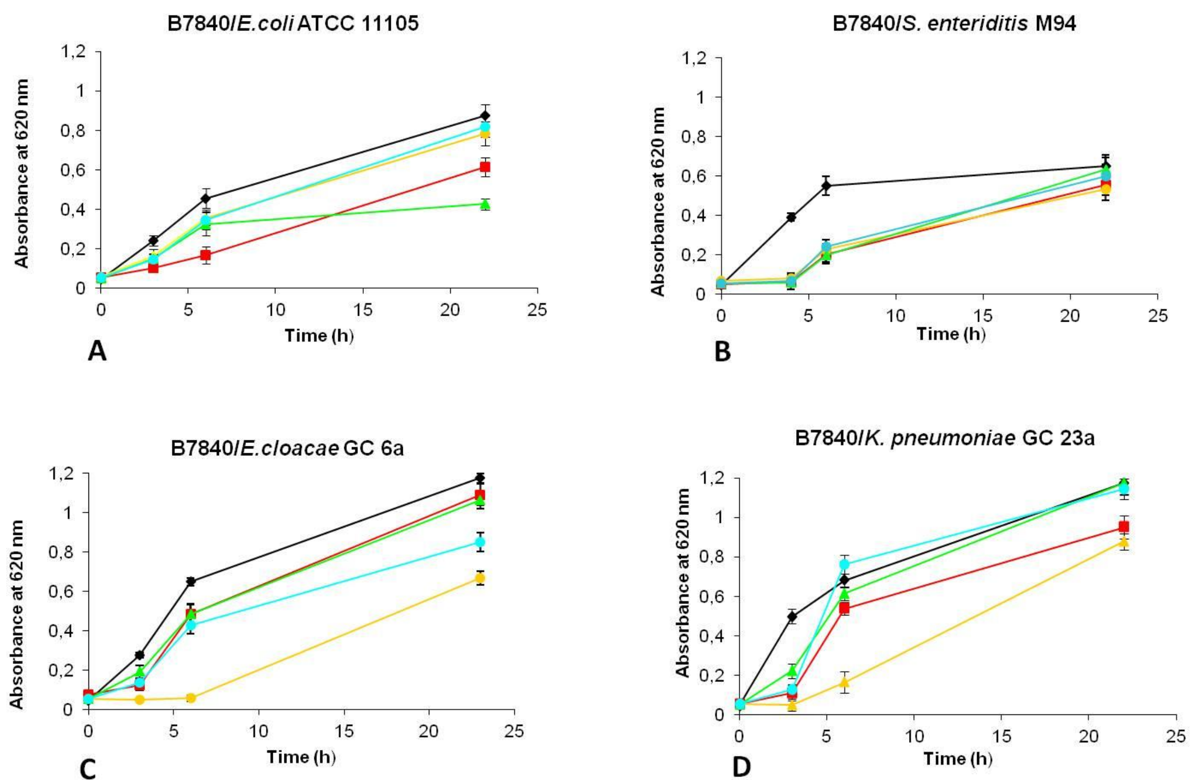


Figure 20 Effect of culture supernatants (CS) and neutralized culture supernatants (NCS) of of *B. breve* B7840 on the growth of *E. coli* ATCC 11105 (A), *S. enteriditis* M94 (B), *E. cloacae* GC6a (C), *K. pneumoniae* GC23a (D), control with 50 µl NB (black), 25 µl CS (red), 50 µl CS (yellow) , Δ 25 µl NCS (green), 50 µl NCS (light blue).

12.3 Genotypic characterization of the *Bifidobacterium* strains

The selected 16 strains were identified and classified at the species level using the ERIC-PCR approach proposed by Ventura et al., (2003). An accurate clustering and identification of the strains was achieved comparing ERIC-PCR banding patterns of the strains used in this work with those retrieved from reference strains (**Figure 21a** and **21b**).

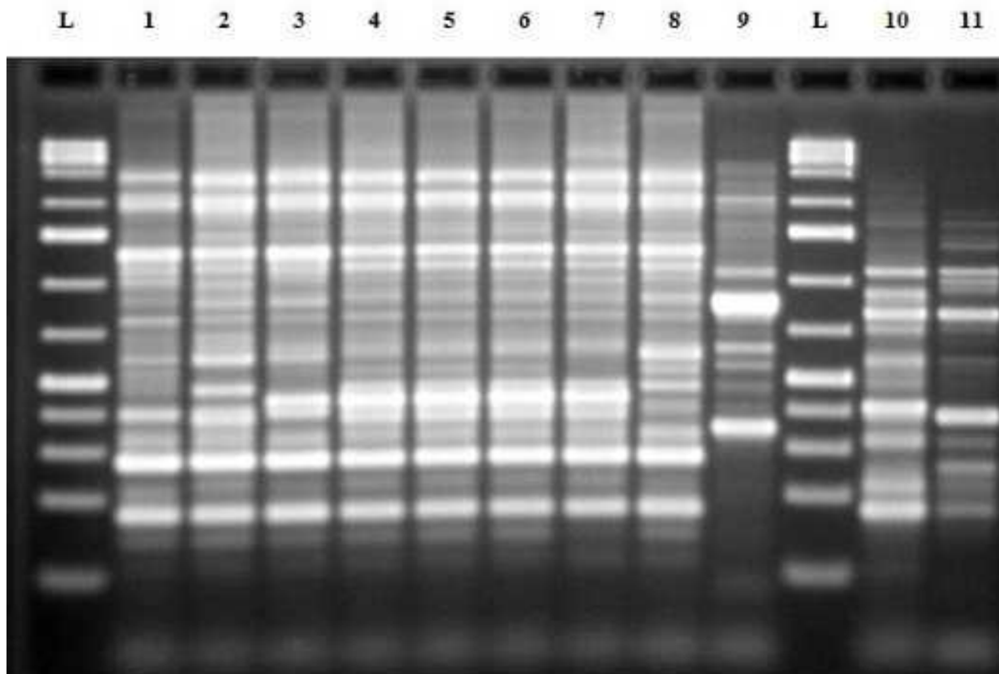


Figure 21a ERIC-PCR patterns 3 different species of *Bifidobacterium*: *B. breve, B. longum* subsp.*infantis, B bifidum*. Lane L,100bp DNA Ladder (Fermentas), lane 1,Re1 (*B. breve* ATCC 15700T, type strain), lane 2, B632, lane 3, B2021, lane 4, B2150, lane 5, B2195, lane 6, B2274, lane 7, B7840, lane 8, B7847, lane 9, Re6 (*B. longum* subsp.*infantis* ATCC 15697T, type strain), lane 10, MB28 (*B. bifidum* DSM 20456T, type strain), lane11, B2091.

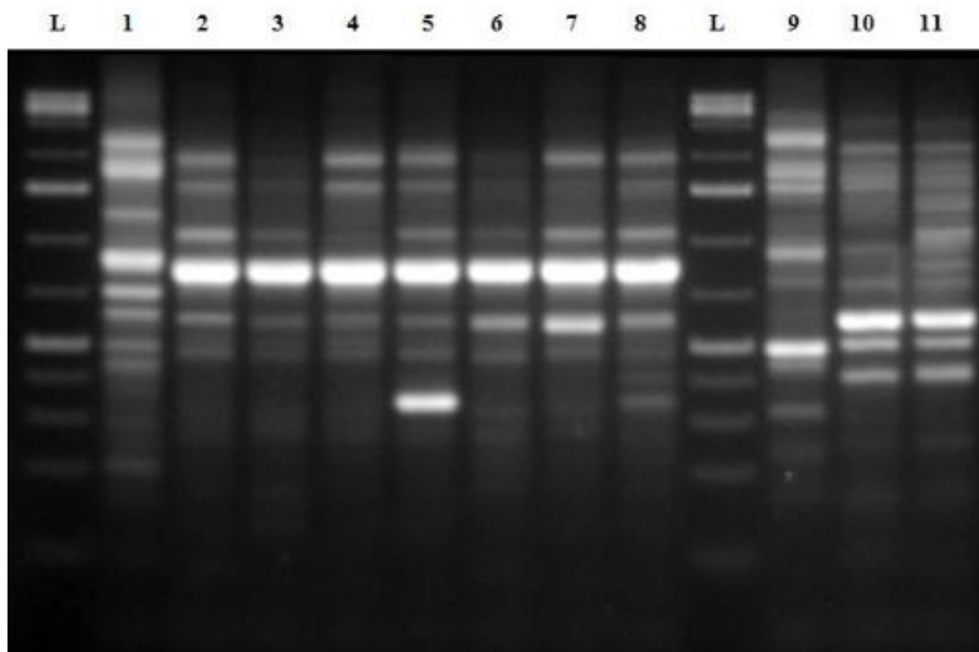


Figure 21b. ERIC-PCR patterns of 3 different species of *Bifidobacterium*: *B. longum* subsp.*longum, B catenulatum*, *B. pseudocatenulatum.* Lane L,100bp DNA Ladder (Fermentas), lane 1,Re11 (*B. longum* subsp. *longum* ATCC 15707^{T}, type strain), lane 2, RE12, lane 3, B1412, lane4, B1975, lane 5, B2055, lane 6, B2101, lane 7, B2192, lane 8, B7958, lane 9, B669 (*B.catenulatum* ATCC 27539^{T} ,type strain), lane 10, B8452, lane 11, B1279 (*B. pseudocatenulatum*ATCC 27917^{T},type strain).

The CA and the scatterplot projections of variables (strains) and cases (ERIC-PCR bands) on the first two dimension evidenced four main clustering groups corresponding to different type strains (**Figure 22**). One group was formed by the *B. pseudocatenulatum* type strain (ATCC 27917^{T}) and the B8452 strain: it was the most divergent cluster due to the exclusive presence of 8 DNA fragments. A second main group clustered with the *B. longum* strains including the *B. longum* subsp. *longum* and the *B. longum* subsp. *infantis* type strains: 6 strains clustered close to *longum* subspecies and were therefore identified as *B. longum* subsp. *longum* (B1412, B1975, B2055, B2101, B2192, B7958, Re12). A third cluster grouped with the *B. breve* type strain (B632, B2021, B2150, B2274, B2195, B7840, B7847). Finally, the B2091 strain clustered close to the *B. bifidum* type strain.

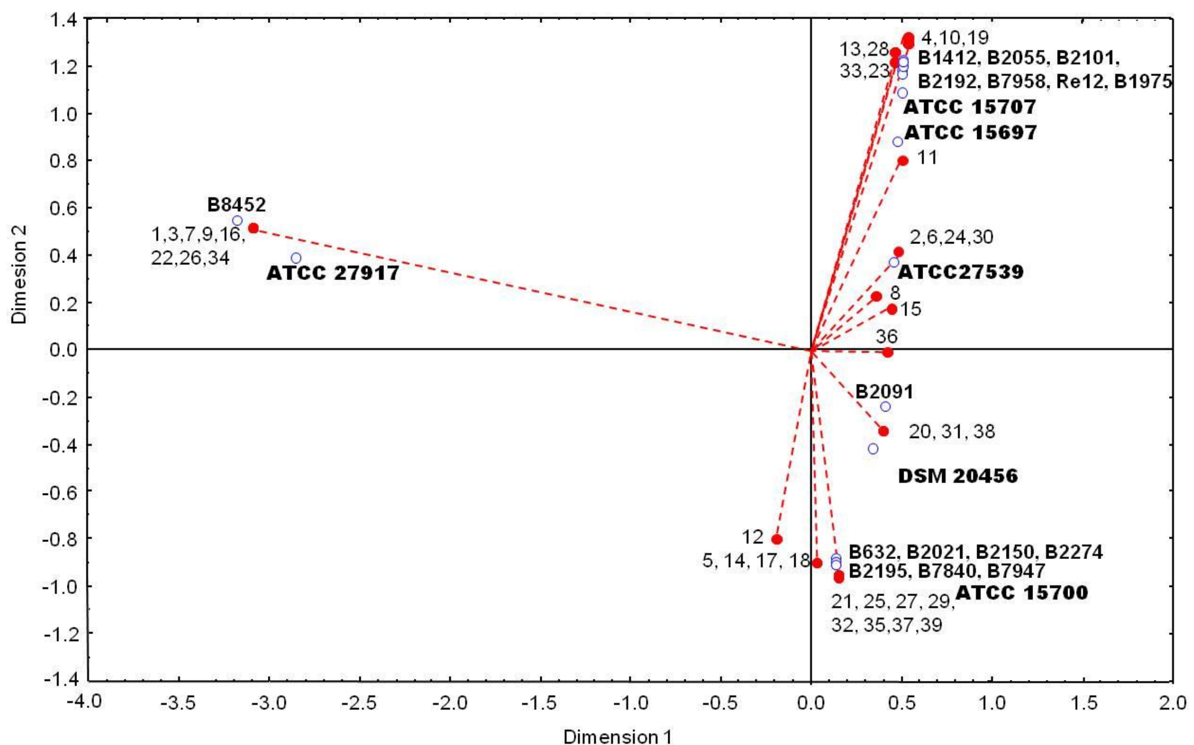


Figure 22 Relationships established among *Bifidobacterium* strains by means of CA based on ERIC-PCR band patterns. Numbers correspond to fingerprinting DNA fragments obtained after agarose gel electrophoresis following ERIC-PCR.

To confirm the results obtained with ERIC-PCR, the strain identification was compared with species-specific standard PCR . 16S targeted species specific primers allowed to confirm the *Bifidobacterium* identification at the species level, except for the *B. pseudocatenulatum* strain which was only inserted in the "*catenulatum* group" with this technique.

12.4 Antibiotic resistance profiles

12.4.1 Minimal inhibitory concentration (MIC)

The determination of the antibiotic resistance of bifidobacteria and LAB is an important issue, considering that these probiotics are often co-administered with antibiotics. On the other hand, probiotics could represent a potential source for the spread of antibiotic genes. The determination of the resistance or sensitivity to certain antibiotics is recommended by EFSA.

The resistance or sensitivity of the selected 16 strains to 12 antibiotics and the relative MIC values obtained are shown in Table 3. All the strains were found to be sensitive to chloramphenicol, erythromycin, vancomycin (apart from B2091) and gentamycin according to most recent EFSA guidelines (EFSA, 2008). Moreover, most of the strains were sensitive to tetracycline except a few strains (B2055, B2150, B2195,B2274, B7840, B7958). All the strains were resistant to ampicillin and the majority of them to kanamycin (except B1412). 9 strains out of 16 were resistant to streptomycin. Regarding cefuroxime, ceftriaxone and clarithromycin , whose breakpoints are not present in the mentioned EFSA guidelines, the majority of the strains presented low MIC values so they can be considered sentitive to them. All the strains but one (B632) presented a high MIC value for amoxicillin.

Table 16 MIC of various antibiotics of the selected strains. Strains are characterized as sentive (S) or resistant (R) according to the breakpoints defined by EFSA (2008)

Strain	Minimum Inhibitory Concentration (µg/ml)																							
	AMP (2)		CHL (4)		ERY (0.5)		TET (8)		VAN (2)		KAN (8)		STR (128)		GEN (64)		CEFU *		AMO *		CEFT *		CLA *	
Re12	≥256	R	2	S	0.5	S	4	S	2	S	64	R	32	S	8	S	8	ND	≥256	ND	2	ND	2	ND
B632	≥256	R	4	S	0.1	S	1	S	0.5	S	64	R	≥256	R	32	S	8	ND	2	ND	4	ND	2	ND
B1412	≥256	R	4	S	0.1	S	2	S	2	S	4	S	≥256	R	32	S	2	ND	≥256	ND	2	ND	2	ND
B1975	≥256	R	4	S	0.5	S	2	S	2	S	32	R	32	S	32	S	2	ND	≥256	ND	2	ND	2	ND
B2021	≥256	R	4	S	0.25	S	2	S	2	S	≥256	R	≥256	R	32	S	8	ND	≥256	ND	2	ND	2	ND
B2055	≥256	R	4	S	0.5	S	64	R	2	S	32	R	128	S	16	S	4	ND	≥256	ND	4	ND	2	ND
B2091	≥256	R	4	S	0.5	S	8	S	≥4	R	≥256	R	≥256	R	64	S	8	ND	≥256	ND	2	ND	2	ND
B2101	≥256	R	2	S	0.5	S	8	S	2	S	128	R	64	S	64	S	2	ND	≥256	ND	2	ND	2	ND
B2150	≥256	R	4	S	0.5	S	64	R	0.5	S	≥256	R	≥256	R	64	S	32	ND	≥256	ND	8	ND	2	ND
B2192	≥256	R	4	S	0.5	S	2	S	2	S	≥256	R	64	S	32	S	8	ND	≥256	ND	4	ND	2	ND
B2195	≥256	R	4	S	0.5	S	32	R	2	S	128	R	≥256	R	64	S	16	ND	≥256	ND	8	ND	2	ND
B2274	≥256	R	4	S	0.5	S	32	R	2	S	≥256	R	≥256	R	32	S	32	ND	≥256	ND	8	ND	2	ND
B7840	≥256	R	2	S	0.5	S	32	R	2	S	≥256	R	16	S	32	S	32	ND	≥256	ND	8	ND	2	ND
B7947	≥256	R	2	S	0.25	S	2	S	2	S	≥256	R	256	R	32	S	≥256	ND	≥256	ND	2	ND	2	ND
B7958	≥256	R	4	S	0.5	S	32	R	2	S	128	R	128	S	32	S	4	ND	≥256	ND	2	ND	2	ND
B8452	≥256	R	2	S	0.1	S	2	S	0.5	S	≥256	R	≥256	R	32	S	2	ND	≥256	ND	2	ND	2	ND

AMP = ampicillin, CHL = chloramphenicol, ERY = erythromycin, TET = tetracyclin, VAN = vancomycin, KAN = kanamycin, STR = streptomycin, GEN = gentamycin, CEFU = cefuroxime, AMO = amoxicillin, CEFT = ceftriaxone, CLA = clarithromycin

12.4.2 Screening of resistance genes

The screening of the resistance genes via PCR amplification of known genes in the 16 strains of bifidobacteria allowed to detect the *tet* (W) amplicon only in two (B2274 and B7840) of the 6 tetracyclin resistance strains, whereas none of them was positive to *tet*(M) and *tet* (O). Only three strains (B1975, B2192, B7947) out of the 15 resistant to kanamycin were positive to *aph*(3'')-III amplification, whereas *aph*(3'')-I and *aph*(3'')-II were not amplified in any strain. With regard to the β-lactam (β-lac) resistance determinants, almost all the tested strains carried blaCTX-M-g1 apart from B2021, B2101, B2150, B2274, 7958 (**Figure 27**). No strains were found to be positive to the amplification of the *aad*A and *aad*E streptomycin resistance genes (**Table 17**) .

Table 17 Positive PCR for resistance genes in the 16 *Bifidobacterium* strains and relative control strains

Strain	TET	KAN	STR	B-LAC
Re12				blaCTX-M-g1
B632				blaCTX-M-g1
B1412				blaCTX-M-g1
B1975		*aph*(3'')-III		blaCTX-M-g1
B2021				
B2055				blaCTX-M-g1
B2091				blaCTX-M-g1
B2101				
B2150				
B2192		*aph*(3'')-III		blaCTX-M-g1
B2195				blaCTX-M-g1
B2274	*tet* (W)			
B7840	*tet* (W)			blaCTX-M-g1
B7947		*aph*(3'')-III		blaCTX-M-g1
B7958				blaCTX-M-g1
B8452				
L9*		*aph*(3'')-III	*aad*A, *aad*E	
Ru424*	*tet* (W)			

*strains used as positive control

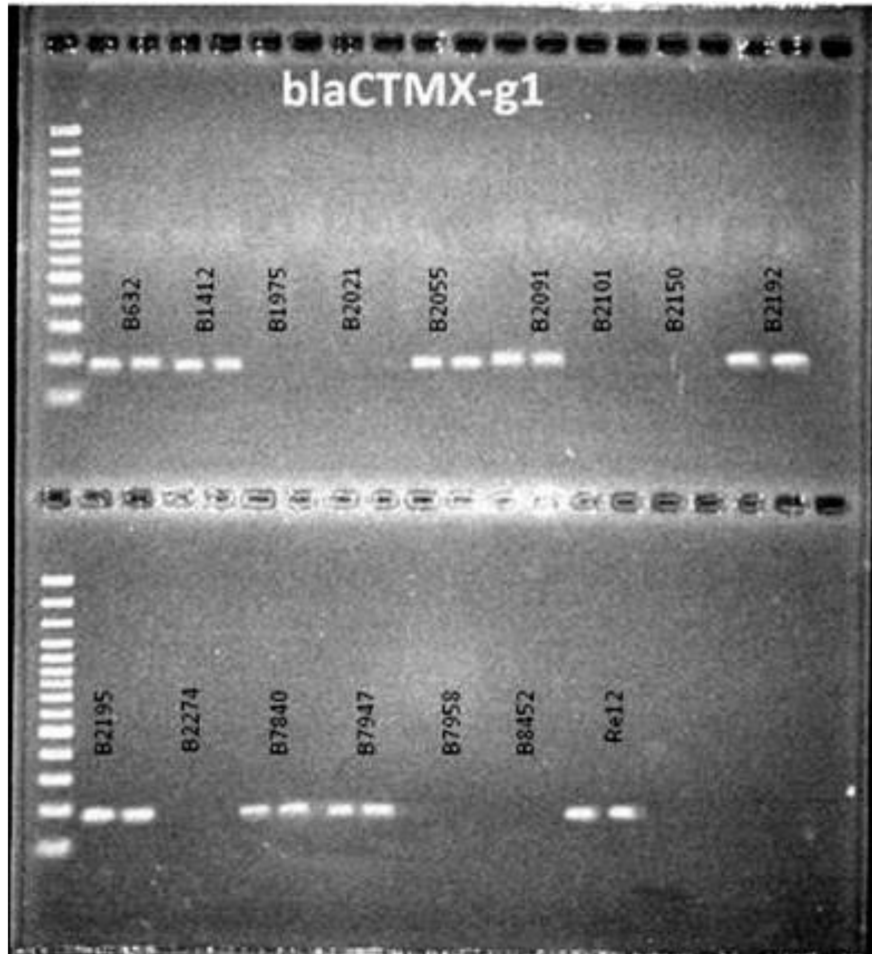


Figure 27 PCR products of blaCTX-M-g1 gene obtained for 9 *Bifidobacterium* strains.

12.4.3 Plamid detection

One of the main mechanism of horizontal transfer of genes in bacteria in natural environment is believed to be conjugation. Therefore, it is known that plasmids play an important role in the dissemination of antimicrobial resistance. This is the reason why the presence of plasmids was checked. Plasmids were detected only in *B.longum* subsp. *longum* B2192 strains, which was found to posses two plasmids (**Figura 28**).

Figure 28 Plasmid profiles patterns of 9 *Bifidobacterium* strains: lane a, B2192, lane b, B2399, lane c,B632, lane d, B1412, lane e, B1975, lane g, B2055, lane h, B2192, lane i, B2274, lane l, B7840, lane m, B8452. λ/ hindIII DNA ladder (Fermentas).

12.5 *In-vitro* interaction between *Bifidobacterium* strains and human cells

12.5.1 Cytotoxicity and adhesion

Cytotoxicy assays showed that a number of strains (B1412, B2021, B2091, B2101, B2150, B2192, B7947, B7958 and Re12) at the bacterial concentration of 10^7 CFU/mL after 90 min incubation exherted a cytotoxic effect to the H4 monolayers higher than the control strain LGG (P<0.05). Referring to TLT monolayers, LGG resulted the most cytotoxic strain (**Figura 23**), nevertheless, a consistent number of strains showed a low reduction of viability of TLT cells when compared to untreated cells, although data were not statistically significant. However, it has to be considered that a direct contact between the content of the intestinal lumen with macrophages is not an *in vivo* real condition. Only three *B. breve* strains B632, B2274, B7840, *B. longum* B2055 and *B. pseudocatenulatum* B8452 showed positive effects on both cell monolayers, in particular B632 and B2274 seemed to increase the viability of cells after the exposure (**Figure 29**).

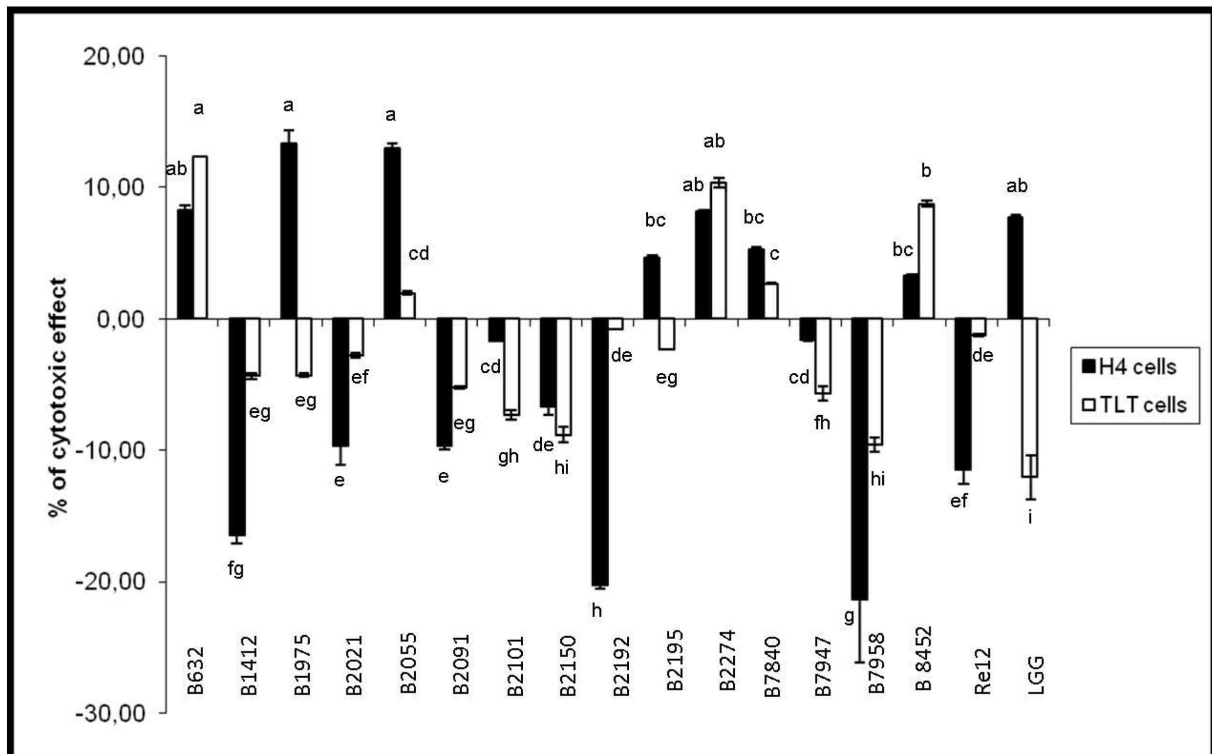


Figure 29 Cytotoxic effect of 16 *Bifidobacterium* spp. strains on the H4 and TLT cell monolayers. The LGG strain is used as control. Results are expressed as the average of three independent experiments (± SD). Mean with different letters are significantly different at P< 0.05.

All strains showed a good ability to adhere to polarized human epithelial H4 cells and TLT macrophages. **Figure 30** reports the % of adherent bacterial cells compared to initial inoculum.

B. breve B632, *B. pseudocatenulatum* B8452 and *B. longum* B2192 showed a higher attachment to H4 cells with respect to the reference strain LGG whereas the majority of *Bifidobacterium* strains presented an adhesion capability comparable to LGG or slightly higher. The strain which showed a reduced capacity of attachment were *B. longum* B1975, B2091and B7958 (**Figure 30**).

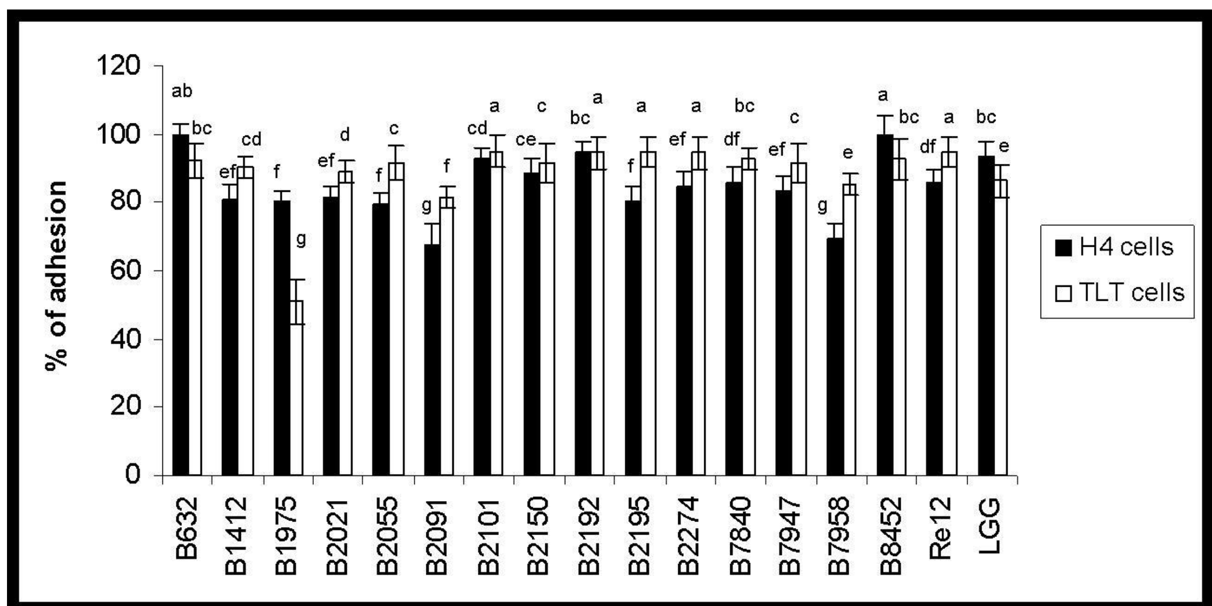


Figure 30 Adhesion of 16 *Bifidobacterium* strains and the LGG strain (used as control) to H4 and TLT cell monolayers. Results are expressed as the average of three independent experiments (± SD). Mean with different letters are significantly different at P< 0.05.

15.5.2 Stimulation of cell activity: mitochondrial activity, production of reactive oxygen species and of interleukin

The results of the mitochondrial activity enhancement with the MTT assay are shown in **Figure 31**. The mitochondrial dehydrogenase activity of H4 and TLT cell lines increased after exposure to *B. breve* B632 and B 2195 strains at the concentration of 1×10^7 CFU/ml. However, the percentage of stimulation obtained for most of the strains was higher than that obtained with the LGG strain. In addition, the stimulation was as negative as those obtained with the *S. enteriditis* and *E. coli* strains (i.e. potential pathogens), in particular these two microorganism showed a market negative effect on

the cellular mitochondria. Macrophages cell line TLT, resulted to be generally more feeble than ephithelial cells, indeed only B632 was able to strongly stimulate the activity of mitochondrial dehydrogenase of macrophages; while only a slight enhancement was obtained with B2021 and B2274.

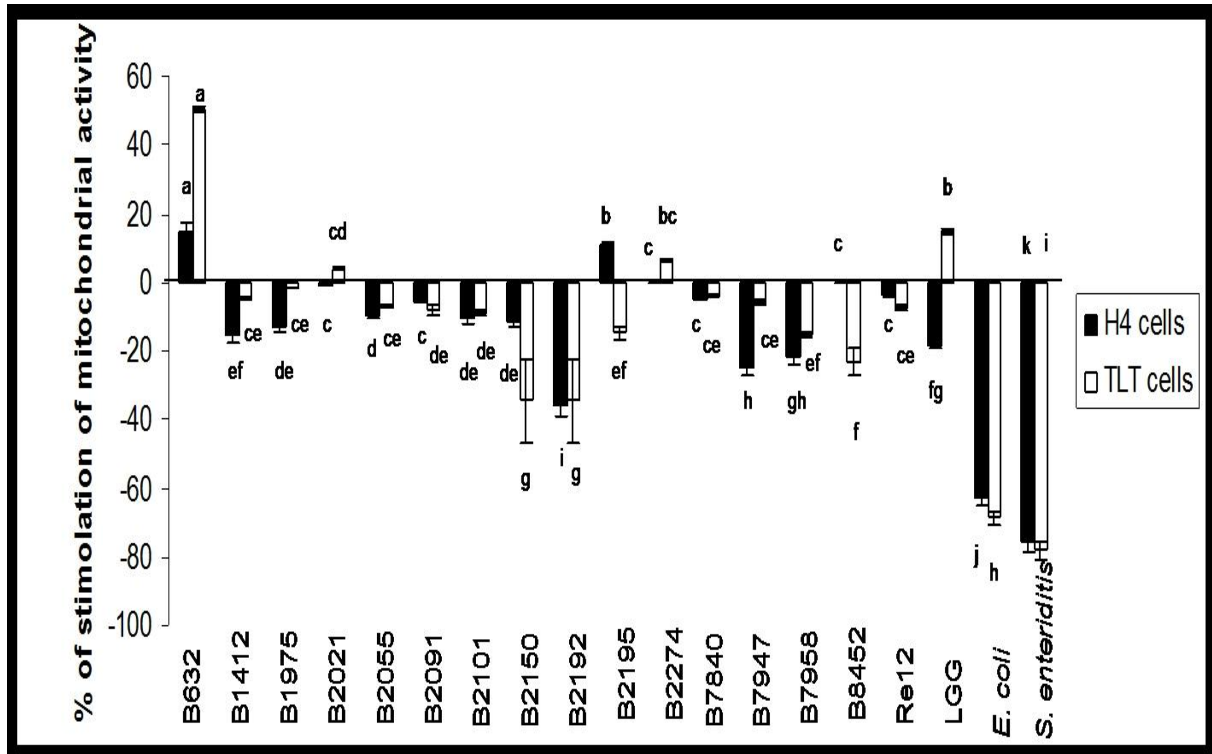


Figure 31 Effect of 16 *Bifidobacterium* spp. strains on the mitochondrial dehydrogenase activity of H4 and TLT cell monolayers. The LGG strain, *E. coli* and *S. enteriditis* are used as control. Results are expressed as the average of three independent experiments (± SD). Mean with different letters are significantly different at $P< 0.05$.

Among the 16 *Bifidobacterium* strains, applied at the concentration of 10^7 CFU/mL on H4 cell line, only B2274 induced an increase of NO production statistically higher than the reference strain LGG. Except for B632, B2091 and B7840 strains, the remaining *Bifidobacterium* strains exhibited a lower stimulation effect on NO production than LGG strain. As concerns the stimulation of NO production on TLT cell line, the strongest induction was observed for B1412 strain (approximately 5 times higher with respect to LGG strain). A moderate increase of NO production, comparable with that observed for LGG strain, was reported for B2091, B2274, B7840, B7947 and B7958 strains. Twelve out of 16 *Bifidobacterium* strains stimulated H_2O_2 production from H4

cell lines, while all *Bifidobacterium* induced an increase of hydrogen peroxide from TLT cell lines. The B1412, B2021, B2055, B2150 and B2195 strains induced an increase of H_2O_2 production on H4 cell line statistically higher than LGG strain. In contrast, only one strain (B7947) was more efficacious in stimulating H_2O_2 production of TLT cell line than LGG. *E. coli* and *S. enteriditis*, used as potential entheropatogens, induced the strongest stimulation of ROS production (ie nitric oxide, hydrogen peroxide) in both H4 and TLT cell lines (**Table 18**).

Table 18 ROS production (nitric oxide, hydrogen peroxide) by different intestinal cell lines (H4, TLT) as a function of the stimulation from different bacterial strains. The results are expressed as mean ratios (%) of ROS production with respect to controls (intestinal cell lines not exposed to bacterial strains). Mean values followed by different letters (between brackets) are statistically different at P < 0.001.

Strains	**Nitric oxide**		**Hydrogen peroxide**	
	H4	**TLT**	**H4**	**TLT**
B632	7.40 (ce)	7.78 (dg)	6.53 (dh)	27.27 (fg)
B1412	-2.36 (e)	61.71 (c)	25.63 (c)	27.29 (fg)
B1975	-4.32 (e)	-3.35 (eh)	2.51 (fi)	20.51 (gi)
B2021	-5.62 (e)	-1.12 (dh)	9.55 (df)	14.10 (ij)
B2055	-3.01 (e)	-2.60 (dh)	17.59 (ce)	23.94 (fh)
B2091	2.19 (ce)	1.86 (dh)	4.23 (fi)	10.08 (jk)
B2101	-4.32 (e)	-5.58 (fh)	-4.86 (hj)	16.24 (ij)
B2150	-1.06 (de)	-2.60 (eh)	8.54 (df)	28.79 (ef)
B2192	-0.41 (de)	-7.06 (gh)	-14.57 (j)	6.48 (kl)
B2195	-6.92 (e)	-1.86 (dh)	18.39 (cd)	28.64 (ef)
B2274	18.14 (c)	2.60 (dh)	3.52 (fi)	34.62 (e)
B7840	15.21 (cd)	8.89 (df)	8.17 (dg)	28.64 (ef)
B7947	1.54 (de)	10.78 (de)	1.97 (fi)	54.55 (c)
B7958	-8.22 (e)	1.86 (dh)	-11.28 (j)	17.78 (hi)
B8452	-6.27 (e)	-8.55 (h)	5.93 (eh)	29.06 (ef)
Re12	-4.97 (e)	0.37 (dh)	-6.61 (ij)	2.10 (l)
LGG	3.49 (ce)	12.58 (d)	-4.02 (gj)	46.15 (d)
E. coli	223.87 (a)	199.68 (a)	138.33 (a)	146.87 (a)
S. enteriditis	160.51 (b)	143.67 (b)	111.15 (b)	123.67 (b)
P	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
LSD	16.56	14.89	12.29	7.12

Dot-blot was performed to determine the presence of pro-inflammatory cytochine IL-6 in cell free culture supernatants after exposure of cells to the bacteria for 24 h. A notable production of IL-6 was achieved by with H4 cells with all bacteria except for *B. longum*

subsp. *longum* B1412. The highest IL-6 production was noted for B632 and B2055 (**Figure 32**). IL-6 production of TLT cells were obtained after exposure to the majority of the strains apart from B1412, B2150, B2195, B7840, B7947, B8452. However, a general greater production of IL-6 by H4 with respect to TLT resulted from the intesity of the dots.

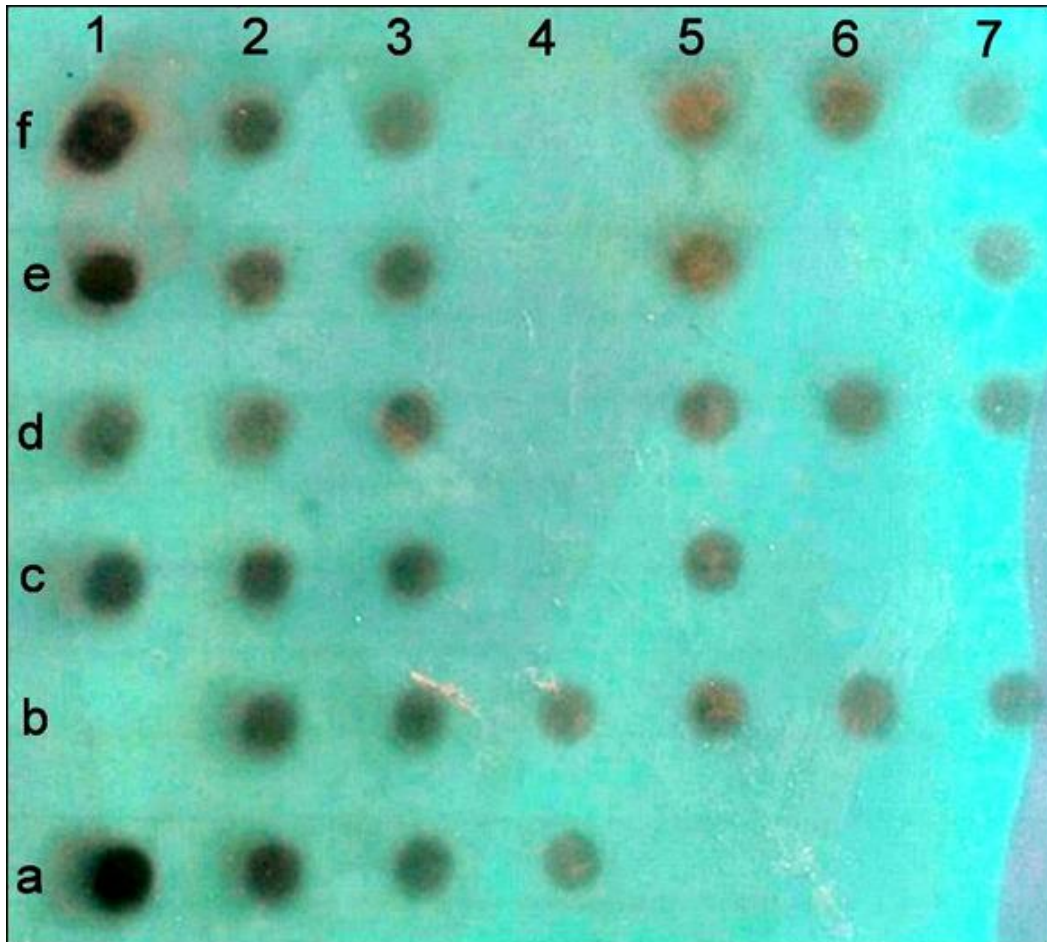


Figure 32 Dot-blot of IL-6 detection. The experiment was performed with 16 *Bifidobacterium* spp. strains. LGG and *L. casei* Shirota were used as positive controls; negative controls do not have any applied *Bifidobacterium* strain (H4 or TLT untreated cells).

1a: B632/H4, 1b:B1412/H4, 1c: B1975/H4, 1d: B2021/H4,1e:B2055/H4, 1f:2101/H4,
2a:B2150/H4, 2b:B2192/H4, 2c:B2195/H4, 2d:B2274/H4, 2e:B7840/H4, 2f:7958/H4,
3a:B8452/H4, 3b:Re12/H4, 3c:B2091/H4, 3d:B7947/H4, 3e:LGG/H4, 3f: LGG /H4,
4a: *L.casei* Shirota /H4, 4b: *L.casei* Shirota /H4, 4c: neg control/H4, 4d:neg control/H4, 4e: neg. control/TLT, 4f: neg control/TLT,
5a:B1412/TLT,5b:B2091/TLT,5c:B1975/TLT, 5d: B2021/TLT, 5e:B2055/TLT, 5f:2101/TLT.
6a:B2150/TLT,6b:B2192/TLT,6c:B2195/TLT,6d:B2274/TLT, 6e:B7840/TLT, 6f: B632/TLT,
7a:B7947/TLT,7b:B7958/TLT,7c: B8452/TLT,7d: Re12/TLT,7e: LGG/TLT,7f: *L. casei* Shirota /TLT.

12.6 Selection of the best probiotic strains with the use of a synthetic index

A global evaluation of all the results obtained during the first phase of the work has been carried out in order to establish which *Bifidobacterium* strains, among the 16, are the more suitable to be used as probiotics for human use. In this respect, the outputs of each analysis were transformed into relative percentages and summerized into a data matrix (**Table 19**). The criterion adopted involved the use of different correction factors based on the importance of each parameter on for the evaluation of the *Bifidobacterium* strains; since the safety of use must be a pre-requisite for a new probiotic, no correction factor was used for the cytotoxic assays and antibiotic resistance evaluations. Furthermore, the same criterion was applied in the case of the antimicrobial activity against the enteric pathogens and the evaluation of adhesion to gut cells, which are the most important functional aspects for the purpose of the work. For all the other results (MTT assays, ROS production) a correction factor of 0.5 was applied in the calculation. The matrix thus completed allowed to calculate a synthetic index. The strains with the highest synthetic index were selected, i.e. B632, B2274, and B7840. In addition, the B1975 strain was also chosen for further studies because of its high synthetic index and its high antimicrobial activity against potential pathogens.

Table 19 Selection of the most promising *Bifidobacterium* strains with the determination of a synthetic index. The outputs of each analysis (spot agar tests, antibiotic resistance or sensitivity assay, cytotoxicity test, adhesion assay, mitochondrial dehydrogenase activity, NO and H_2O_2 production) were transformed into relative percentages by giving the 100 value to the strain showing the best performance in each test. A correction factor of 0.5 was given to the mitochondrial dehydrogenase activity, NO and H_2O_2 production tests. The IL-6 production was not considered in this test as it is not a quantitative test.

Strain	Spot agar test/1[a]	Spot agar test/2[b]	Antibiotic resistance[c]	Cyto-toxicity H4 cells	Cyto-toxicity TLT cells	Adhesion H4 cells	Adhesion TLT cells	MTT[e] assay H4 cells	MTT[e] assay TLT cells	NO production H4 cells	NO production TLT cells	H_2O_2 production H4 cells	H_2O_2 production TLT cells	Synthetic index
B632	93	75	37.5	62	100	99	96	50	50	20	6	13	25	727
B1412	100	75	50	-124	-35	79	94	-51	-4	-7	50	50	25	302
B1975	77	95	50	100	-8	88	63	-43	-1	-12	-3	5	19	430
B2021	77	75	37.5	-73	-23	80	92	-2	4	-15	-1	19	13	283
B2055	50	70	50	75	13	75	92	-33	-7	-8	-2	34	22	430
B2091	56	80	25	-41	-16	66	85	-19	-8	6	2	8	9	252
B2101	86	60	25	-12	-59	90	100	-37	-9	-12	-5	-9	15	234
B2150	77	40	37.5	-50	-71	86	94	-39	-34	-3	-2	17	26	178
B2192	93	60	50	-204	-6	93	100	-120	-34	-1	-6	-28	6	1
B2195	77	80	25	30	-19	76	98	34	-15	-19	-2	36	26	428
B2274	93	85	25	62	84	82	100	-2	7	50	2	7	32	626
B7840	75	85	37.5	40	22	85	97	-16	-4	42	7	16	26	512
B7947	43	100	37.5	-12	-46	80	95	-84	-6	4	9	4	50	275
B7958	73	30	37.5	-160	-78	69	89	-73	-15	-23	2	-22	16	-55
B8452	34	75	37.5	25	71	100	95	-1	-23	-17	-7	12	27	426
Re12	79	75	50	-86	-10	84	99	-12	-7	-14	0	-13	2	248

[a] data obtained against *E. coli*, *E. cloacae, K. pneumoniae*, *S. enteriditis* (Table 1) were considered
[b] data obtained against *C. jejuni* and *C. difficile* (Table 3) were considered
[c] the number of antibiotic resistances (according to EFSA guidelines, EFSA 2008) was considered (Table 4). The absence of antibiotic resistances correspond to the maximum value (100).
[e] the MTT assay regards data on mitochondrial dehydrogenase activity stimulati

12.7 Transferability of antibiotic resistance traits

The capability of B632, B1975, B2274 and B7840 of transferring the antibiotic resistance traits to other was studied according to the scheme proposed in Table. As recipient strains, some *Bifidobacterium* spp. strains and lactic acid bacteria (*Lactobacillus plantarum* PCS22, *Lactobacillus casei* L9 and *Enterococcus faecium* PCD71) were used. The choice of recipient strains was done considering their sensitivity to the antibiotics used in the assay. No recipient strains could receive the antibiotic resistance trait from all the donors and, in addition, no spontaneous mutants of the 4 donor strains was detected (**Table 20**).

Table 20 Evaluation of the transferability of the antibiotic resistance traits from *B. breve* B632, B2274 and B7840 and *B. longum* B1975 to selected recipient strains

Donor strain	Antibiotic resistance assayed*	Recipient strain(s)	Selection plates	Strains with acquired antibiotic resistance (CFU/ml)	Spontaneous mutants (CFU/ml)
B632	Ampicillin (blaCTX-M-g1)	ATCC 27536	TPY+ AMP + TET	-	-
	Ampicillin	PCS22	MRS + AMP + aerobiosis**	-	-
	Kanamycin	B1412	TPY+ KAN + AMO	-	-
	Streptomycin	B7840	TPY+ STR + TET	-	-
B1975	Ampicillin (blaCTX-M-g1)	ATCC 27536	TPY+ AMP + TET	-	-
	Ampicillin	PCS22	MRS + AMP + aerobiosis	-	-
	Kanamycin (aph (3'')III)	B1412	TPY+ KAN + STR	-	-
	Amoxicillin (blaCTX-M-g1)	PCD71	MRS + AMP + aerobiosis	-	-
B2274	Ampicillin	PCS22	MRS + AMP + aerobiosis	-	-
	Tetracycline (tetW)	PCD71	MRS + TET + aerobiosis	-	-
	Kanamycin	B1412	TPY + KAN + TRIM	-	-
	Streptomycin	B7840	TPY+ STR + KAN	-	-
	Amoxicillin	B632	TPY+ AMO + TRIM	-	-
	Amoxicillin	PCD71	MRS + AMO + aerobiosis	-	-
B7840	Ampicillin (blaCTX-M-g1)	PCD733B	TPY+ AMP + STR	-	-
	Ampicillin	PCS22	MRS + AMP + aerobiosis	-	-
	Tetracycline (tetW)	B632	TPY+ TET + STR	-	-
	Tetracycline	PCD71	MRS + TET + aerobiosis	-	-
	Kanamycin	B1412	TPY+ KAN + STR	-	-
	Amoxicillin (blaCTX-M-g1)	B632	TPY+ AMO + STR	-	-
	Amoxicillin	PCD71	MRS + AMO + aerobiosis	-	-

* the resistance genes indicated in brackets has been identified by PCR
** plates were incubated in aerobic conditions to allow the growth only of lactic acid bacteria
AMO = amoxicillin, AMP = ampicillin, CEFT = ceftriaxone, CEFU = cefuroxime, CHL = chloramphenicol, CLA = clarithromycin, ERY = erythromycin, KAN = kanamycin, GEN = gentamycin, STR = streptomycin, TET = tetracyclin, TRIM = Trimethoprim, VAN = vancomycin.

Chapter 13. Evaluation of the most effective prebiotic fiber

13.1 Prebiotic Activity Assay

Prebiotic fibers, often employed in human and animal diet, such as FOS, GOS and inulin, were used within this work together with less common polysaccharides derived from plants such as PHGG and arabinogalactans.

The experiment was performed only with the 4 strains that were found to have the best probiotic properties in the previously performed) experiments (*B. breve* B632, B2274, B7840 and *B. longum* B1975).

The first set of prebiotic fibers tested (Synergy, Raftiline, Beneo, Actilight, Vivinal), highlighted the different behaviour of four *Bifidobacterium* strains; which is not surprising considering that early studies on prebiotics reported that carbohydrate utilization pattern differs greatly among *Bifidobacterium* species and inside each species, among different strains (Crociani et al., 1994). A common feature of the 4 assayed strains was that they could grow well on Vivinal, Actilight and Beneo. However, differences among the strains were observed; the growth of B632 (**Figure 33A**) was mainly sustained by Beneo (i.e. a oligofructose DP< 10) giving an increase inA620 of 1.12 ± 0.03 after 48 hours of incubation. Beneo also supported the growth of B2274 (**Figure 33C**).

Interestingly, the galactooligosaccharide Vivinal, together with the fructooligosaccharide Actilight, were the substrates which best supported the growth of the four strains. On the other hand Synergy and Raftiline (i.e. a inulin DP > 23) substained the growth less than glucose. B7840 could grow on there prebiotic fibers worse than on glucose (**Figure 33D**).

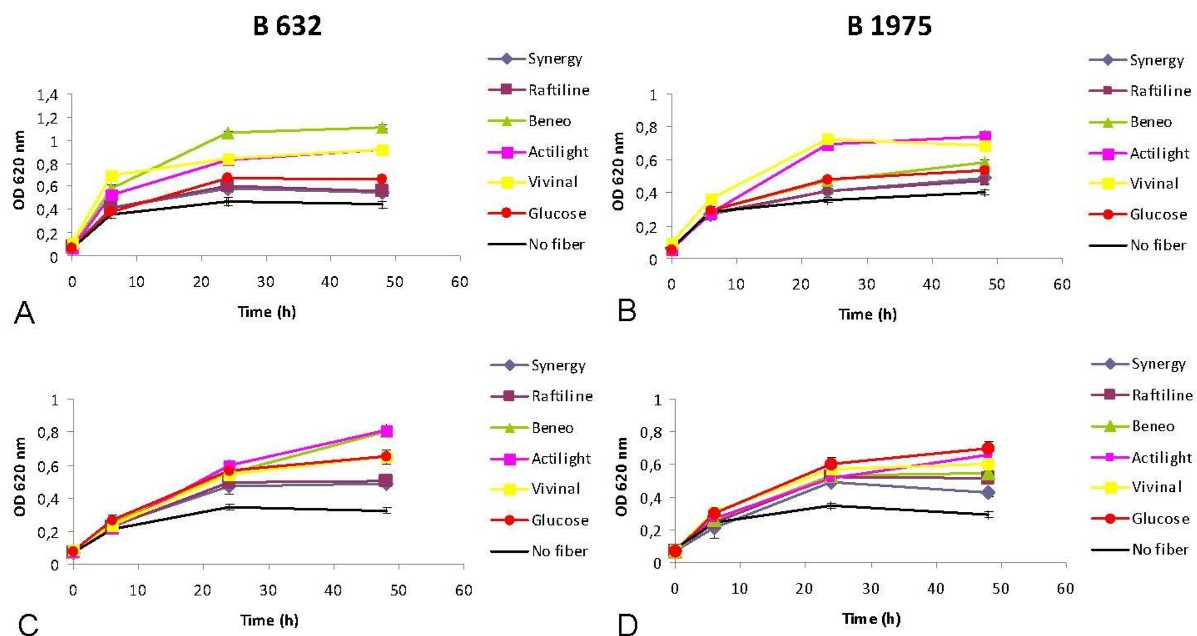


Figure 33 Growth curves of B632, B1975, B2274, B7840 strains using prebiotic fibers (Synergy, Raftiline, Beneo, Actilight, Vivinal) as sole carbon source. Glucose used as positive control for the growth.

As regards the second set of prebiotics (Larch fiber, FOS, BioEcolians, CUP-oligo, Benefibra, Frutafit), most of them supported the growth of the strains and, in several cases growth was better that on glucose. B 632 **(Figure 34A)** could grow on BioEcolians (a glucooligosaccharides), CUP and FOS better that on glucose. BioEcolians was also the best prebiotic for B1975 **(Figure 34B)**, although growth was lower than on glucose. FOS could sustain the growth of B 2274 **(Figure 34C)** and B 7840 **(Figure 34D)** better than glucose. The growth of the latter strain was also very good on BioEcolians and CUP-oligo.

A good prebiotic fiber should be selectively fermented by probiotics, while it should not sustain growth of potentially harmful bacteria. Therefore, the capability of a mixture of coliform strains potentially involved in enteric diseases in newborns (*E. coli*, *K. pneumonia* and *E. cloacae*) of growing on the same fibers was assayed. Differently from the *Bifidobacterium* strains, the coliform mixture could not grow on any of the first set of fibers used in this work (**Figure 35A**) whereas it could grow well on glucose.

On the contrary, FOS could sustain the growth the coliform mixture better that glucose (**Figure 35B**).

B632 showed a great capability to grow on the most of the prebiotic substrates assayed.

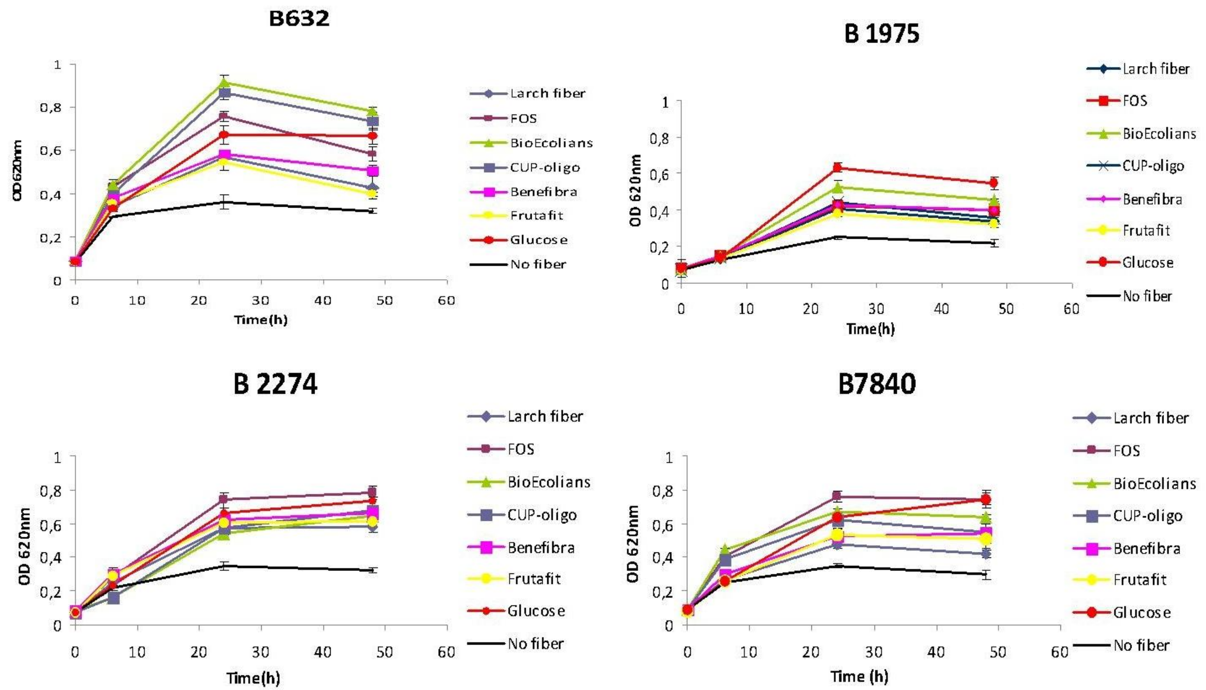


Figure 34 Growth curves of B632, B1975, B2274, B7840 strains using prebiotic fibers (Larch fiber, FOS, BioEcolians, CUP-oligo, Benefibra, Frutafit) as sole carbon source. Glucose used as positive control for the growth.

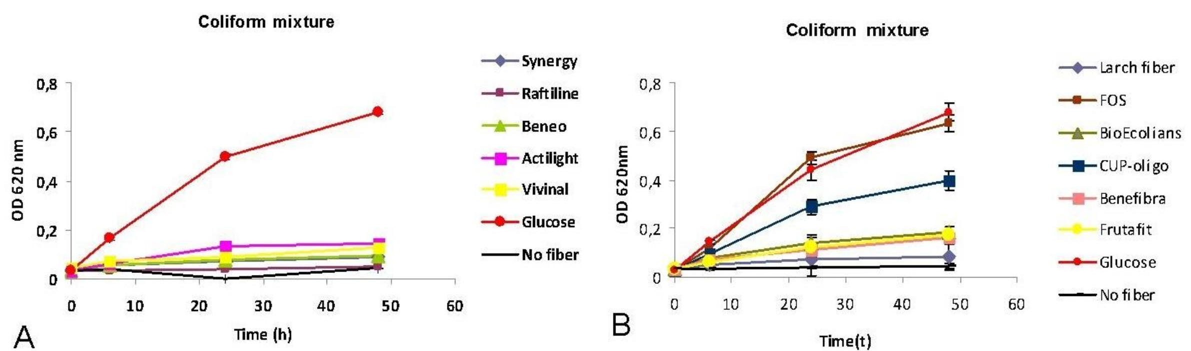


Figure 35 Growth curves of coliform microorganism mixture (*Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae*, 1:1:1) using prebiotic fibers as sole carbon source. Glucose used as positive control for the growth.

Several authors (Huebner et al., 2007; Depeint et al., 2008; Marotti et al., 2012) have proposed to evaluate the efficacy of a prebiotic fiber by comparing its capability of sustaining the growth of a probiotic strain with that of glucose and with *E. coli* or a mixture of selected bacterial strains. The prebiotic activity scores have been calculated as decribed in chapter 10, taking as reference strains the 1:1:1 mixture of the coliforms (**Figure 36**). Beneo, Actilight, Vivinal and BioEcolians presented the highest prebiotic score, although they do not supported the growth of all the *Bifidobacterium* strains at the same level. On the contrary, Synergy and Raftiline showed similar values of prebiotic scores for all the 4 bifidobacteria. Finally, it is interesting to note that FOS which greatly supported the *Bifidobacterium* growth (**Figura 34**), showed the lowest prebiotic score,due to its capability of sustaining the enteric mixture growth.

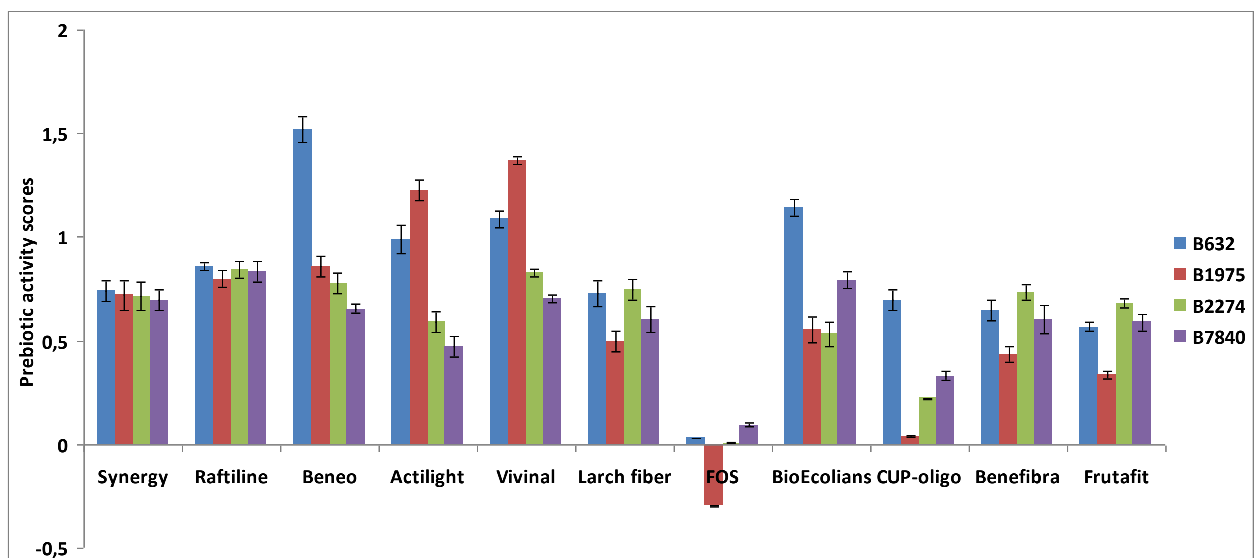


Figure 36 Prebiotic activity scores calculated by using the mean of prebiotic scores obtained from the four different Bifidobacterium strains (B632, B1975, B2274, B7840) and enteric mixture (*Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae*, 1:1:1) as target. Values are mean of three different repications ± standard deviations.

To better understand these results, growth curves have been also obtained with each single enteric strains: *E. coli* (i.e. the coliform present at the highest concentration in newborns), *E.cloacae* and *K. pneumoniae* (**Figure 37 and 38**).

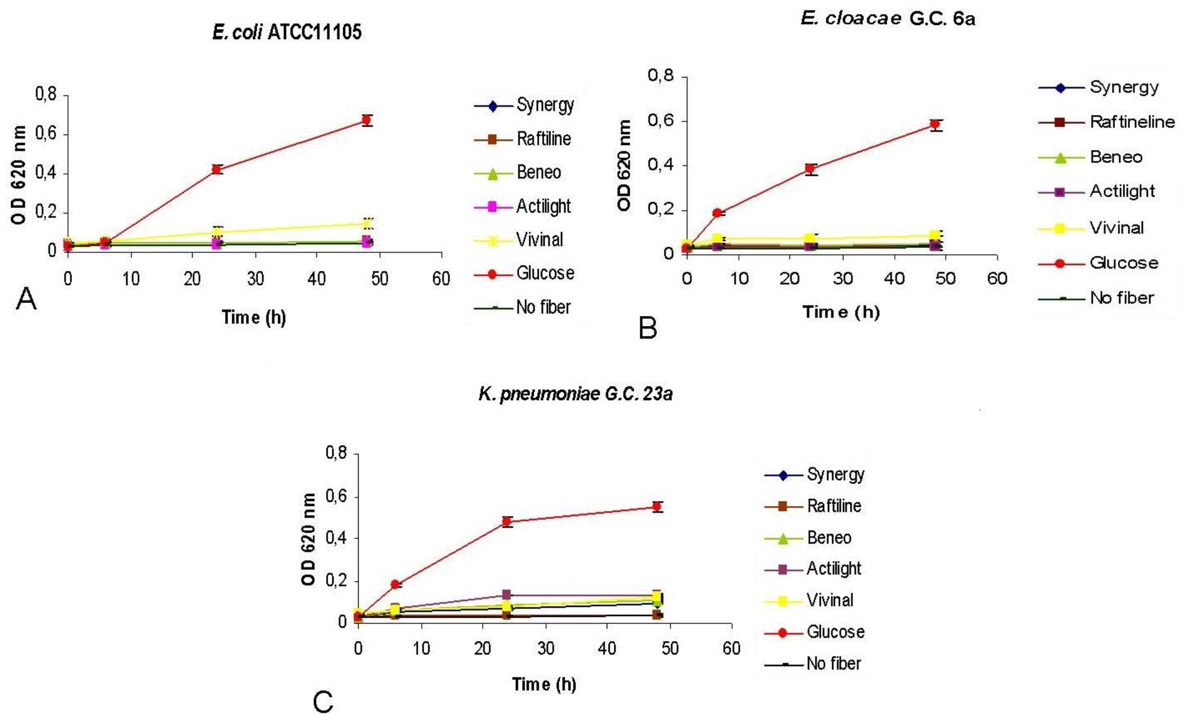


Figure 37 Growth curves of *Escherichia coli* (A), *Klebsiella pneumoniae* (B), *Enterobacter cloacae* (C) using prebiotic fibers (Synergy, Raftiline, Beneo, Actilight, Vivinal) as sole carbon source. Glucose used as positive control for the growth.

Growth curves of coliform bacteria evidenced that none of the first set of prebiotic fibers (Synergy, Raftiline, Beneo, Actilight, Vivinal) used sustained the growth of such bacteria a part from glucose (**Table 37**). As regards to the second set of prebiotics (Larch fiber, FOS, BioEcolians, CUP-oligo, Benefibra, Frutafit), two of them, FOS and CUP-oligo, supported the growth of the strains and, in particular FOS supported the growth of *K. pneumoniae*, better than glucose (**Figure 38 C**). This result explained the low prebiotic index of FOS.

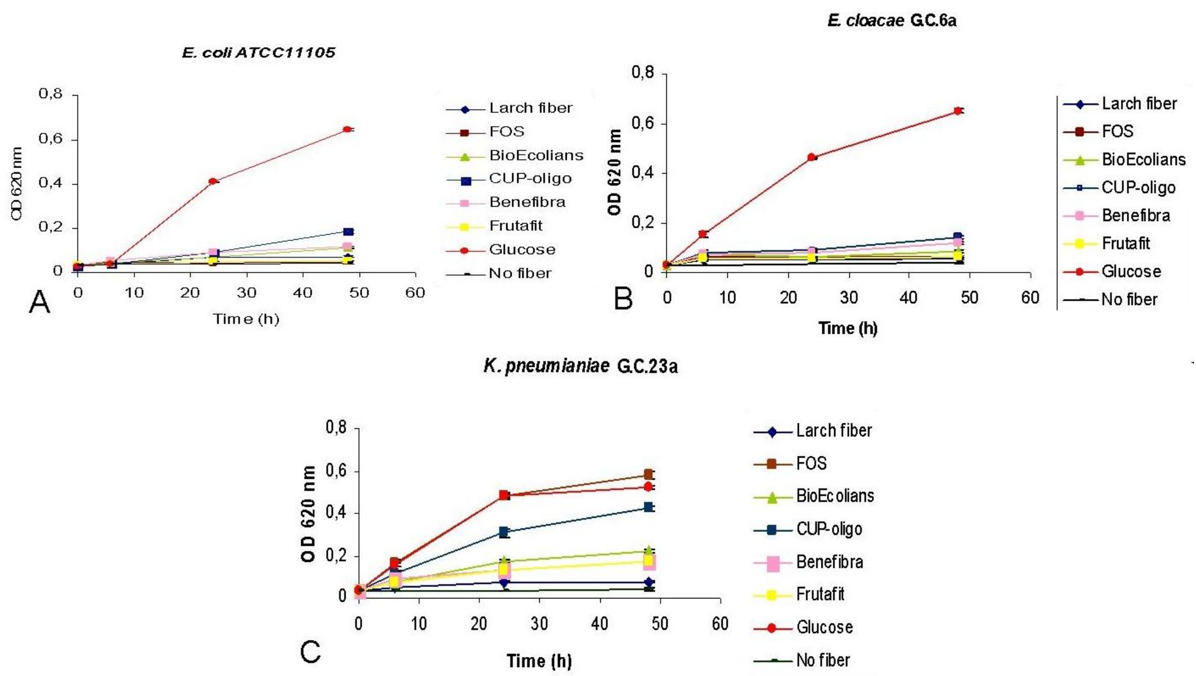


Figure 38 Growth curves of *Escherichia coli* (A), *Klebsiella pneumoniae* (B), *Enterobacter cloacae* (C) using prebiotic fibers (Larch fiber, FOS, BioEcolians, CUP-oligo, Benefibra, Frutafit) as sole carbon source. Glucose used as positive control for the growth.

Chapter 14. Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn microbiota

14.1 Microbiological analysis of newborn fecal samples

To analyse the effects of the maternal antibiotic treatment against *Streptococcus* infection on the intestinal microbiota of the newborns, the quantification of the principal groups of the newborn gut microbiota was carried out. *Lactobacillus* spp., *Bidobacterium* spp., *Bacteroides fragilis* group., *C. difficile* and *E.coli* quantification was obtained with real-time PCR. **Table 22** shows the microbial counts of stool samples of newborns whose mothers were treated with ampicillin and of control samples (i.e. newborns whose mothers were not treated with any antibiotics).

Table 22 Median counts of the different microbial groups analyzed in newborn stool samples expressed as Log (CFU/g of feces) for different microbial groups.

Target	***Bifidobacterium* spp.**		***Lactobacillus* spp.**		***E. coli***		***C. difficile***		***B. fragilis* group**	
Antibiotic treatment	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**
	7.77	6.83	6.40	6.03	10.23	6.04	3.85	3.74	10.24	9.98
	4.12	3.24	6.67	5.40	9.87	8.35	4.80	4.80	10.25	4.86
	7.10	5.53	6.37	6.00	8.79	11.42	3.06	3.72	5.22	11.08
	7.05	5.13	7.93	6.20	10.73	10.35	2.85	3.77	10.53	11.15
	7.90	5.51	5.45	6.02	10.79	11.40	5.46	3.58	8.99	11.34
	7.46	4.97	6.74	5.98	9.74	4.09	3.19	3.54	10.38	4.67
	9.71	3.78	6.37	6.22	9.55	10.35	3.76	3.16	10.19	10.41
	7.57	5.87	5.84	6.82	5.38	5.03	4.02	3.96	9.90	5.09
	9.41	5.47	6.91	6.53	10.38	6.18	3.12	3.48	7.05	5.47
	4.88	6.66	7.07	7.80	5.73	6.40	3.29	3.87	11.16	6.72
	9.08	5.62	5.85	6.63	10.12	5.35	3.89	3.98	10.75	6.66
	4.04	5.31	7.59	6.76	7.35	11.12	3.86	4.18	7.75	10.90
	7.64	5.25	6.16	7.08	6.33	10.63	4.33	4.27	7.00	7.09
	4.46	7.67	6.35	6.29	6.10	10.73	4.47	4.48	6.80	11.07
		7.54		6.71		6.00		4.08		10.39
		4.86		6.45		10.64		3.94		6.76
mean	7.01[a]	5.49[a]	6.55	6.41	8.65	8.39	3.85	3.90	9.01	8.32
sd	1.22	1.13	0.67	0.59	2.02	2.74	0.74	0.43	1.87	2.73

[a] Mean values of *Bifidobacterium* spp. are statistically significant at P< 0.05(*)

The results obtained suggested that most of the microbial genera and species analysed were not affected by the maternal treatment with ampicillin. In particular no variation in the nunber of *Lactobacillus* spp., *C. difficile* and *E.coli* was observed associated to the treatment. However, *E. coli* counts show a wide variability within each group of samples: 5.73-10.79 Log CFU/g in control group and 4.09-11.72 Log CFU/g in treated group. On the contrary, *Lactobacillus* spp. and *C. difficile* counts do not show great variability within and between the two groups.

A slighly lower number of *B. fragilis* was found in the stools of newborns born from treated women (8.32Log CFU/g) with respect to respected to control samples (9.01Log CFU/g), although these data did not result significantly different after statistical analysis. *B.fragilis* group counts found in the two groups were very variable; furthermore a distribution different from the Gaussian one can be hypothesized because the median values (8.53 Log CFU/g and 10.04 Log CFU/g in the treated and control group, respectively), have a greater differences with respect to the two average values (8.32 Log CFU/g and 9.01 Log CFU/g).

The most interesting results obtained were the different counts of *Bifidobacterium* spp. between the two groups of newborns. The maternal treatment with ampicillin against the risk of *Streptococcus* infection resulted to reduce the intestinal colonization of *Bifidobacterium*: 5.49 Log(CFU/g) of treated samples against 7. 01 Log(CFU/g) of control samples. Even if data variability, was wide also in this case, differences resultedstatistically significant at $P < 0.05$. In order to reduce the variability of the population, a wider number of samples is necessary and we are at present going on with newborn stool sampling and analyses.

PART 5: DISCUSSION

Probiotics are increasingly being used for the treatment of diseases and minor gastrointestinal problems in infants. A recent study has evidenced positive effects on infant colics after treatment of newborns with a *L. reuteri* strain (Savino et al., 2010) , whereas no studies have been performed up to now regarding the use of bifidobacteria for this purpose. This work was therefore aimed at the characterization of *Bifidobacterium* spp. strains possessing *in vitro* capabilities of inhibiting the growth of pathogens typical of the infant gastrointestinal tract without exerting toxic activities on the gut epithelium and harmful effects to the host. Moreover, the possibility of stimulating the growth of *Bifidobacterium* strains with the use of a prebiotic fiber was explored with the objective of defining a synbiotic product to be administered to newborns suffering from gastrointestinal problems. The last part of the work has been focused on the exploration of the microbial diversity of 7 day old newborns, whose mother had been subjected to an antibiotic therapy a few hours before the delivery because of a streptococcal infection. These newborns can in fact be a possible target for probiotic treatment.

Chapter 15. Selection and characterization of Bifidobacterium strains

The majority of *Bifidobacterium* spp. strains used in this work derive from infant faeces (Scardovi et al., 1979), i.e. from the source which constitutes the target population of the potential probiotic (Arboleya et al., 2011). Pre-term isolates were also included considering the high stressing environment of the pre-term infant gut, which shows an higher prevalence of *C. difficile* compared with term infants (Penders et al., 2006). Sixteen strains out of the 46 assayed in this study were capable of contrasting the growth of pathogens which are the main cause of infectious diarrhoea of bacterial origin in infants, such as *E. coli*, *S. enteriditis*, *C. difficile* and *C. jejuni* (Rowland, 2008; Van Niel et al., 2002). Moreover, the same *Bifidobacterium* strains showed marked antimicrobial activity against gas producing coliforms isolated from stools of colicky infants. Considering that gas forming coliform concentration is higher in colicky infants

with respect to healthy controls (Savino et al., 2009; Savino et al., 2011), the results obtained are interesting in the perspective of developing a probiotic based therapy for colic treatment in newborns. The number of *Bifidobacterium* strains showing antimicrobial activity was lower by using NCSs. However, this experiment pointed out that at least in some strains, such as *B. breve* B632, the inhibitory activity may not result only from the production of acidic metabolites, but also from the action of other cell excreted metabolites such as bacteriocins. This result represents an interesting starting point for further studies aimed at the characterization of inhibitory molecules in this strain.

A clear taxonomic identification is necessary for the use of a probiotic strain in humans (Arboleya et al., 2011). The genotypic characterization approach used in this work allowed to cluster the majority of the 16 strains into two species, i.e. *B. breve* and *B. longum* subsp. *longum*, whereas only two strains were clustered within the *B. pseudocatenulatum* and *B. bifidum* species. The results of this analysis confirm that *B. pseudocatenulatum* and *B. catenulatum*, which are indistinguishable by standard PCR, can be easily and quickly distinguished via the ERIC-PCR approach (Ventura et al., 2004). The strain B1412, which has been previously identified as *B. longum* subsp. *infantis*, has now been included in the *longum* subspecies.

According to the most recent EFSA guidelines (EFSA, 2008), the spread of resistance to antimicrobials in bacteria requires the examination of the sensitivity/resistance to a number of antibiotics for potential probiotic strains as well as the risks of the resistance traits to be transferred to other bacteria. Except for a number of antibiotics for which the majority of the assayed *Bifidobacterium* strains are resistant, such as ampicillin, kanamycin and amoxicillin, or sensitive, such as chloramphenicol, erythromycin and vancomycin, there is a great variability among strains also belonging to the same species, as already evidenced in the literature (Masco et al., 2006; Ammor et al., 2008). Intrinsic resistance to aminoglycosides such as streptomycin and kanamycin is commonly present in bifidobacteria (D'Aimmo et al., 2007); however, information on streptomycin resistance genes is limited for *Bifidobacterium* strains (Kiwaki and Sato, 2009). Aminoglycoside resistance genes, including *aadE* which was evidenced in a *B. longum* strain (Ouoba et al., 2008), were not found in the genome of the assayed strains as well as the kanamycin resistance genes *aph* (Ouoba et al., 2008). Conversely, all the

strains were sensitive to the aminoglycoside gentamycin, in agreement with the data present in the literature on bifidobacteria (Ammor et al., 2008). The MICs for tetracycline obtained for most of the tested strains suggested the presences of tetracycline resistance genes. *Tet* genes, coding for ribosomal protection protein, are involved in resistance to tetracycline and *tet*(M) and *tet*(W) have been exclusively found in bifidobacteria (Aires et al., 2007). However, only two of the assayed strains, *B. breve* B2274 and B7840, presented the *tet*(W) amplicon. Bifidobacteria are usually susceptible to β-lactams, such as ampicillin and amoxicillin (Ammor et al., 2008; Matto et al., 2007), whereas the majority of the strains considered in this analysis are resistant. Consequently, resistance to some β-lactams can be considered an acquired resistance and therefore has the potential for lateral spread (EFSA, 2008). There is very little information on the mechanisms responsible for horizontal gene transfer in anaerobic gut bacteria like bifidobacteria; however, the most widespread is the conjugation of plasmids carrying the antibiotic resistance genes. All the 16 *Bifidobacterium* spp. strains potentially considered interesting for the aims of this study did not carry any plasmids, although plasmids have been identified in several bifidobacteria species and strains (Ventura et al., 2008). However, other genetic mechanisms can influence the likelihood of genetic transfer (Burrus and Waldor, 2003), such as transposons, which can carry resistance genes and can move from chromosome to plasmids and vice-versa, thereby increasing the mobility of these genes. Therefore, the transferability of the antibiotic resistance traits to *Bifidobacterium* spp. strains and lactic acid bacteria was assayed in the four strains which were considered the most interesting ones for the aim of this study (*B. breve* B632, B2274, B7840 and *B. longum* subsp. *longum* B1975) and the results allowed to conclude that there was no transfer of the antibiotic resistances neither to the bifidobacteria nor to the lactic acid bacteria assayed.

Finally, adhesion and cytotoxic effects to human cells of the 16 putative probiotic strains were evaluated using non tumorigenic cell lines, which have already been used as a reliable *in vitro* method for the selection of lactic acid bacteria with potential probiotic properties (Maragkoudakis et al., 2010; Nissen et al., 2009), but have never been tested with *Bifidobacterium* spp. strains. This part of the work has been wholly performed at the Department of Biochemistry, Faculty of Medicine, University of Maribor under the scientific supervision of professor Avrelija Cencic. It is well assessed

that the phenotype of tumorigenic cell lines traditionally used for this purpose distinguishes them profoundly from the normal gut epithelium (Tremblay and Slutsky, 2007). The ability to adhere to the intestinal epithelium is one of the most important features as it allows to persist in the colon preventing the elimination by peristalsis and the adhesion of pathogenic bacteria. All the tested bacteria showed a good adhesion to both cell types, epithelial cells and macrophages. Furthermore, adhesion cannot singly determine the biological activity of these putative probiotic strains. It is a combination of different factors which determines epithelial integrity, viability and immuno response. Treatments with *B. breve* B632, B2274 and B7840, *B. longum* B2055, *B. pseudocatenulatum* B8452 manifested no cytotoxicity over H4 and TLT cell lines at the concentration of 10^7 CFU/mL. In addition *B. breve* B632 and B2274 at the same concentration were able to increase the metabolic activity of cell mitochondria. These results indicate that these strains are not harmful when exposed to a healthy intestine. Most of the tested strains increase the production of ROS in small intestinal epithelial cells and in macrophages. The ability of probiotic bacteria to induce NO secretion from intestinal epithelium may offer a significant contribution to prevent the enteric pathogens from infecting the host. The ability to stimulate NO production in eukaryotic cells is not a common ability of the genera *Lactobacillus* and *Bifidocbacterium,* but rather of individual strains (Pipenbaher et al., 2009). Furthermore, most of the bacterial strains tested induced H_2O_2 release in both types of cells. Moderate production of H_2O_2 and NO induced by probiotics could have a beneficial effect in maintaining a balance and increasing resistance to infections. However, it should be noted that high concentration of H_2O_2 and NO, as displayed by potential enteropathogens such as *E. coli* and *S. enteriditis* (Table 5), can cause tissue injury, disseminated intravascular coagulation and shock (Park et al., 1999). Last but not least, there is extensive evidence that cytokines play a pivotal roles in host defence, inflammatory response and autoimmune disease (Park et al., 1999). Therefore, IL-6 production is likely to be a good indicator of a degree of endothelial cells activation. In the present work exposure of H4 and TLT cells to *Bifidobacterium* and *Lactobacillus* strains resulted in marked increase of IL-6 production. In conclusion, the large array of aspects examined in the first part of the study and summarized in table 19 with the calculation of the synthetic index, has allowed the identification of 4 *Bifidobacterium* strains, *B. breve* B632, ,

B2274, B7840 and *B. longum* subsp. *longum* B1975, as potential probiotics for the treatment of enteric disorders in newborns such as infantile colics or as preventive agents for infantile diarrhoea of bacterial origin. They both 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. These four strains have been deposited to a international strain collection with the following accession numbers: DSM 24706 (*B. breve* B632), DSM 24707 (*B. breve* B2274), DSM 24708 (*B. breve* B7840) and DSM 24709 B1975 (*B. longum* subsp. *longum*). Studies are currently being performed in order to develop suitable ways of administering the selected probiotic strains to newborns with the aim of planning a validation clinical trial.

Chapter 16. Evaluation of the most effective prebiotic fiber

A second part of the study has regarded the selection of a prebiotic fiber with the aim of preparing a synbiotic product to be administered to newborns. The interaction between gut microbiota and human milk has drawn attention to the bifidogenic effect of nutritional supplement and bifidogenicity has become a essential characteristic of the prebiotic concept (Saavedra and Tschernia, 2007). It has been reported that the supplementation of infant formula with specific oligosaccharides stimulates the growth of bifidobacteria in the intestine resembling the effect of breast-feeding (Boehm and Moro, 2008). For this purpose a wide range of different polysaccharide fibers has been analysed in order to establish which of them better supported the growth of the 4 bifidobacteria selected in the first part of the work. The results of prebiotic activity assays suggested that 1 GOS formulations (Vivinal GOS), 2 FOS formulation (Actilight 950P and FOS provided by Probiotical SpA) greatly stimulated the growth of the majority of the strains. In addition, Beneo HSI (inulin), BioEcolians (glucooligosaccharide) and CUP-oligo (GOS) showed a high prebiotic activity toward specific strains, the major effects were exerted on B632. According to the data present in literature the prebiotic properties of galactooligosaccharides are already well known and they are mainly due to the fact that galactooligosaccharides mime the activity of the components of human milk; for this reason they are often added to infant milk formulas

(Macfarlane et al 2008). Prebiotic effects of inulin and oligofructose were evidenced in *in vivo* trials (Kolida et al., 2007); however, our results on fructooligosaccharides and inulin are in agreement with those obtained by other Authors (Rossi et al., 2005) that sustain the thesis that bifidobacteria prefer short chain oligofructose to long chain fructooligosaccharides such as inulin with a high DP. That explains why long chain polysaccharides such as Raftiline were difficultly fermentated by the bifidobacteria tested in this work. It has also been assessed that only a few number of *Bifidobacterium* strains produce extracellular hydrolytic enzymes necessary for fructooligosaccharides fermentation (Perrin et al., 2001). However, carbohydrates have a positive prebiotic activity score if they are metabolized by probiotics but not by other intestinal bacteria. As defined by Huebner et al. (2007), the prebiotic activity reflects the ability of a given substrate to support the growth of a beneficial microorganism relative to other microorganisms and relative to growth on a non-prebiotic substrate, such as glucose. FOS formulation, provided from Probiotical SpA, showed a low prebiotic index, this is due to the fact they support the growth of *K.pneumoniae*, one of the microorganisms used in the coliform mixture. However, it has to be considered that *in vivo* real condition *K. pneumoniae* is not a predominant species in infant microbiota (Savino et al 2009) and therefore it is difficult that it may become the predominant species in the gut.
Bioecolians showed also a high prebiotic activity score comparable to Beneo HSI, Actilight 950P and Vivinal GOS, in particular for the B632 strain.
Therefore, considering the results obtained both in the first phase of this work and in this section, it may be concluded that a synbiotic product for newborn use may be composed of the *B. breve* strain coupled to one of the following fiber: Beneo HSI, Actilight 950P, Vivinal GOS or BioEcolians.
In vitro fermentation studies in a chemostat, capable of controlling pH of the medium, are being planned to discriminate the growth performance of the 4 best fibers.

Chapter 17. Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn microbiota

The last part of the work has considered a possible target for the probiotic strains selected in this work. The analysis has been conducted in collaboration with the Neonatal Intensive Care Unit (Sant'Orsola Hospital, Bologna).The study has regarded the quantification of the main microbial groups present in newborns (*Bifidobacterium* spp., *Lactobacillus* spp., *B. fragilis* group, *C. difficile*, *E.coli*) in 7 day old newborns whose mothers have been subjected to antibiotic prophylaxis against GBS and in controls (i.e. neonates from mothers negative to GBS and therefore not subjected to the prophylaxis). The intrapartum antibiotic prophylaxis of GBS positive women is nowadays routinely used in Europe and USA where it is estimated that about 10% of the mothers result positive to this infection (Ferrieri and Wallen, 2012). Currently, the impact of the antibiotic treatment on the onset of neonatal infections remains unclear and, in particular, the impact of the maternal antibiotic treatment on newborn microbiota composition is totally unknown (Al-Taiar et al., 2011). Previous studies have reported an increase in ampicillin resistant *E.coli* when ampicillin is used in intrapartum prophylaxis (Bizzarro et al ., 2008), however other studies reported that intrapartum ampicillin prophylaxis is associated with decreased early-onset *E.coli* infections (Schrag et al., 2006). Results obtained within this work confirm the great variability existing in the newborn's microbial composition evidenced in several other works (Palmer et al. 2007; Sanders et al. 2010). Microbial counts obtained in this study evidence a great variability in *E.coli*, *B. fragilis* group and *Bifidobacterium* , which are the largest microbial groups in infant microbiota, also in the "control group". The differences in *Lactobacillus* spp. and in the *C. difficile* group were, on the contrary, less marked both within the "treated" and the "control" group.

Only the *Bifidobacterium* counts showed a decrease after antibiotic treatment , this is in agreement with the data reported in literature that suggest that newborn treatment with ampicillin can affect the number of bifidobacteria (Penders et al., 2006; Mangin et al., 2010). Therefore it is conceivable that this may also happen after intrapartum ampicillin prophylaxis. In addition, most of bifidobacteria colonizing the newborn gut derive from the mother and therefore a reduced number of bibidobacteria are available for newborn

colonization after antibiotic treatment. A similar tendency was also observed for the *Bacteroides fragilis* population, although without reaching a statistical significance.
Even though the results obtained are only preliminary, due to the restricted number of samples analyzed up to now, it is possible to speculate that newborns, whose mothers have been subjected to intrapartum antibiotic prophylaxis, can represent a potential target for selectedprobiotic administration. We are now planning a large scale study in which a wider number of newborns are examined and stool samples from the same newborns are withdrawn both at 7 days and at the age of 1 month.

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