University of Modena and Reggio Emilia

Ph.D. SCHOOL OF AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIOTECHNOLOGIES

Capturing the functional differences of the human gut microbiota in health and disease

Tutor

Prof. Maddalena Rossi

PhD. Candidate

Marta Simone

Dean of Ph.D. school

Prof. Andrea Pulvirenti

XXVIII CYCLE

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1. Introduction

1.1 The human intestinal microbiota

The human intestinal microbiota is a complex and specialized system. It is populated by a dense microorganisms concentration that allows to consider the human as "meta-organism" that hosts 10^{14} organisms. It is interesting to think that we have co-evolved with our human archetypal microbiota and our "heirloom" microbiota composition has been passed down as part of our shared human ancestry, but our native ancestral microbiome is now a disappeared ideal; other modern practices for example antibiotics, cesarean section, infant formula feeding, agricultural methods and general sanitary/hygienically preventions have transformed our modern microbiota, compared to that of our ancestors (Merepol and Edward 2015).

The major part of these microorganisms are prokaryotes and they are related to a limited species number. The bacteria are the first components of this ecosystem followed by the *Archeae*, unicellular eukaryotes and viruses with more of 1200 viral genotypes isolated by human fecal samples (Ventura et al., 2011).

The gastrointestinal tract (GIT) is divided in different anatomic regions starting from the oral cavity up to the anal one. The endogenous bacteria are not casually distributed but they are localized in specific areas of the GIT. In particular the stomach and the proximal area of the intestine contain a relatively low number of microorganism (10^3-10^5 cells/g) due to the low pH and due to the rapid flux of nutrients that characterizes this region. Acid-tolerant lactobacilli and streptococci are dominant in the upper part of the intestine. Otherwise, the ileum is characterized by a dense and diversified population (10^8 cells/g). The colon is the principal site of microbial colonization. This body site is inhabited by a high number of strictly anaerobic bacteria (10^{11}-10^{12} cells/g), due to the low turn-over of the nutrients, the low redox potential, the nutrients availability and the absence of oxygen. Species related to *Bacteroides* represent the 30% of the total fecal microbiota. Other dominant groups belong to *Firmicutes* or Gram-positive bacteria at low G-C content which are represented by various *Clostridiales* clusters. Bifidobacteria, enterobacteria and coliforms are few represented (Farnaro et al., 2003). The relative abundances of each species are finely regulated by the bacterial competition for the nutrients and for the adhesion sites.

The microorganisms living in the proximal colon have an higher grow-rate thanks to the high concentration of the available substrates. On the other hand, the availability of these substances is minor in the distal colon and therefore the growth is lower. In particular, carbohydrates and proteins introduced with diet are the carbon and nitrogen sources present in the colon. These molecules escape to the hydrolysis by the host digestive enzymes and reach the colon allowing the

growth of the intestinal bacterial population determining the main fermentation pathways: saccharolytic or proteolytic.

The composition of this ecosystem is constant and preserved at *phylum* level, but it is specific and characteristic of each single individual in terms of the genera and species. If well balanced, this ecosystem positively contributes to the host health and each individual has a specific microbiota that play a pivotal role in the homeostasis of the entire organism.

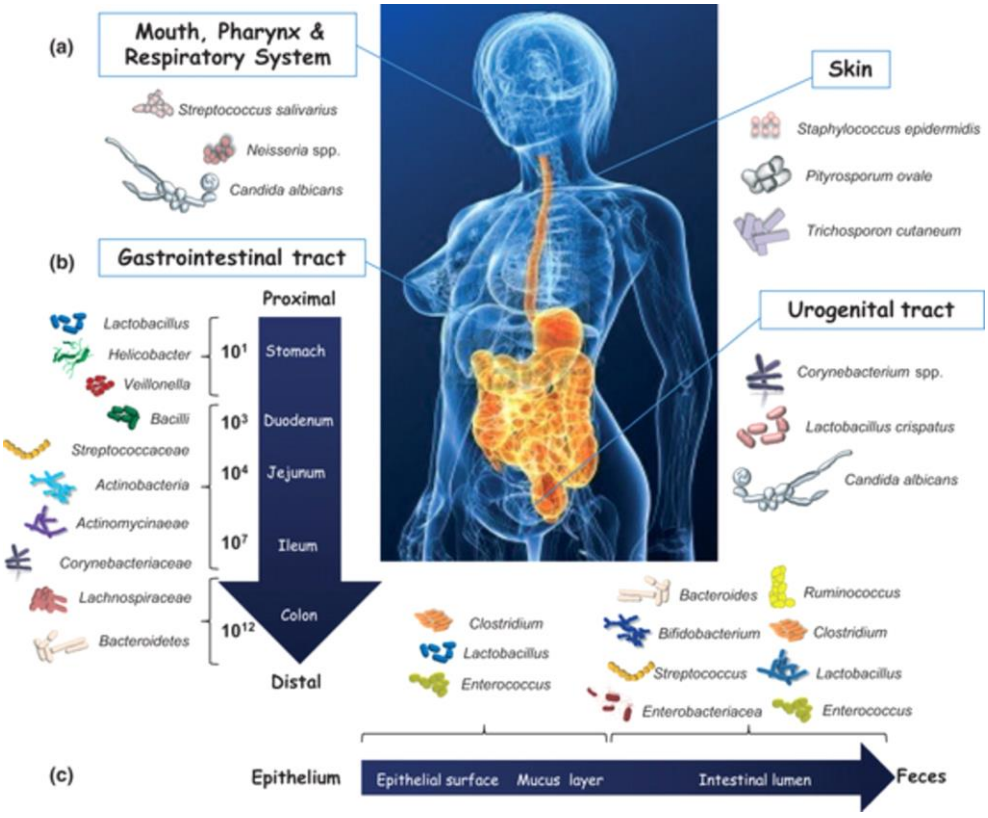


Figure 1: *Representation of the principals gastrointestinal districts and indication of the principals bacteria groups.*

1.2 The intestinal microbiota in early life

The prenatal environment and the GIT in fetus was traditionally thought to be sterile as reported by different studies such as Fanaro et al., 2003, Dominguez-Bello et al., 2010, but several modern researches revealed that the human placenta contains a low abundance of nonpathogenic species belonging mainly to the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria*

phyla. Aagaard et al. (2014) showed that placental species were most similar to those of the human mouth. Comparison between infants born at preterm versus at term revealed distinct patterns of placental colonization, but significant differences were not found between infants born naturally versus by cesarean section (Doyle et al. 2014).

However, during a natural or cesarian birth, the newborn is exposed to the vaginal, fecal and cutaneous microbiota of the mother, with an unavoidable transferring of microorganisms which colonize the oral cavity and the stomach of the infant, influencing the composition of the pre-existent microbiota. Moreover, after the birth the environmental microorganisms (oral and cutaneous ones) are in contact with the newborn through different processes as feeding.

The hygienic preventions adopted in the last years could interfere with this important "contamination" that transfers the microbiota from the mothers to the newborns (Farnaro et al., 2003).

Several studies investigated the dynamics of the second colonization (considering that as reported before the first colonization takes place into the human placenta). A limited quantity of bacteria are present in the GIT after few hours from birth. The first microorganisms that colonize the GIT are *Escherichia coli*, streptococci and staphylococci. This happens because the GIT is characterized by a positive potential redox and by the oxygen presence. The gradual consumption of the oxygen by these bacteria leads to the decrease of intestinal redox potential and allows the formation of the ideal conditions for the growth of the strictly anaerobic bacteria (Orrhage and Nord 1999).

In the breastfed newborns the population of bifidobacteria, lactobacilli and enterococci is consolidated to the detriment of the coliforms after 4 days of life. The *Bacteroidetes* appear for the first time after 10 days from birth and their concentration increase rapidly. The *Bifidobacterium* genus (10^{10}-10^{11} cells/g) is dominant in the breastfeeding phase, but also the *Bacteroidetes* and *Clostridium* are well represented (10^{8}-10^{9} cells/g). After the weaning the bifidobacteria are not the predominant bacterial group and after 2 years a consolidated microbiota similar to the adult one is formed.

The initial composition of the microbiota is simple and subjected to continuous fluctuation (Palmer et al., 2007). Particularly important are some factors related to the host physiology like intestinal pH, temperature, bile salts, peristalsis, immune response and receptors of the intestinal mucosa that influence the tropism and the microbiota-host interactions (Machie et al., 1999).

1.3 How the intestinal microbiota is influenced by the delivery mode and feeding

The environment plays an important role in the colonization of the gastrointestinal tract in the newborn, particularly during the cesarean section where the infant came not in contact with the intestinal and vaginal maternal microorganisms (Biasucci et al., 2010). This environment represents the primary source of bacterial colonization in spite these are characterized by a low ability to colonize the GIT for the first 7 days of life.

In infants born through cesarean section the bifidobacteria or *Bacteroidetes fragilis* concentrations are lower. Bifidobacteria appear only after 3 days (Chen et al., 2007), while the concentrations of *Clostridium difficile* and of *E. coli* are higher (Fouhy et al., 2012). In newborns delivered through caesarian section a higher incidence of *Clostridium perfringens* or *Clostridium tetani* is observed, most probably caused by the contamination of the hospital environment. An increase of the colonization by the *Clostridium difficile* (Penders et al., 2006) that is cause of diarrhea and colics (Fitzpatrick et al., 2008; Thomas et al., 2003) is also observed. Moreover, it has been observed that these infants have an intestinal population strongly influenced by the microorganisms of the mother's skin (e.g stafilococci).

On the contrary, it is evident a vertical transmission of the microorganisms from the mother to the infant during natural birth, with a consequent increase of the lactobacilli after few hours from birth (Dominguez-Bello et al ., 2010).

Another important factor that could influence the composition of the intestinal microbiota is the feeding modality.

The human milk contains the nutrients for the bacterial growth and it is necessary for the consolidation of the intestinal microbiota. It contains complex oligosaccharides (10-12 g/l) that promote the bifidobacteria growth. Moreover, it gives lactoferrin (an antimicrobial protein) and lysozyme, an enzyme that is naturally presents in the milk, in the tears and sweat, which is able to digest the bacterial cellular walls (Fox et al., 2006).

Human milk and cow milk are different for what concern the oligosaccharides composition. The addition of prebiotics to the cow milk compensates for this differences (Manning et al., 2004), determining an increase of the bifidobacteria population in the fecal samples of the newborns formula-fed.

Independently by the feeding modalities, the GIT is initially colonized by streptococci and enterobacteria that create the anaerobic conditions necessary for the growth of specific genera such as *Bacteroidetes* and *Bifidobacterium*.

In breastfed newborns, bifidobacteria can appear after 4 days of age. At the end of the first week, breast milk creates an environment that favors the bifidobacteria growth (Farnaro el al., 2003). Bifidobacteria generally constitute the prevalent population after 6 days (Yoshioka et al., 1991). In another study it was found that at the end of the first week the microbiota of breast-fed infants was rich in bifidobacteria, but in most infants no predominant organisms were found (Roberts et al., 1992). At 1 month of age, the microbiota is stable in the majority of breastfed babies and it is dominated by bifidobacteria while the other bacteria seem to be suppressed. (Yoshioka et al 1983, Balmer et al., 1989, Roberts et al., 1992). In this period the gut of breastfed infant is also populated by enterococci and enterobacteria; clostridia are isolated in low number and *Bacteroidetes* spp. are uncommon residents (Roberts et al., 1992). However by 3 months of age the predominance of bifidobacteria is slightly reduced (Roberts et al., 1992). The strong ability of the bifidobacteria population to compete against other microbial groups has been associated to specific factors occurring in the mother milk, referred to as bifidobacterial factors (Hadault et al., 1996) and also to the introduction of bifidobacteria and other microorganisms (e.g. staphylococci, lactobacilli, and micrococci) with milk itself (Martin et al ., 2003). In breast-fed newborns the population of *E. coli* and clostridia, including *Clostridium difficile*, are lower than in bottle-fed ones. Recent studies have demonstrated that *Ruminococcus* is dominant in the colonization that appear early in life in newborn naturally fed. This bacterium is able to perform positive effect to the host thanks to the production of ruminococcine A, that is a bacteriocin able to decrease the growth of various species of *Clostridium* (Morelli 2008).

In the formula-fed infants the enterobacteria are the prevalent on day 1 as in the breast fed ones; in the next few days enterobacteria tents to remain stable, while bifidobacteria increase very slowly (Yoshioka et al., 1983). After 7 days the fecal microbiota of these babies is without prevalent microorganisms (Roberts et al., 1992) and the bifidobacteria concentration is markedly lower than that encountered in breastfed infants (Yoshioka et al., 1983). Chierici et al. found that in formula-fed infants the occurrence frequency of the bifidobacteria decreased from 71% at the end of the first week to 64% at the end of the first month; at the same times they observed a progressive increase of enterococci and clostridia, the latter rising from 57% to 86% (Chierici et al., 1997).

In bottle fed infants, after 1 and 3 months the microbiota is the similar composition breastfed babies after 7 days. There is no predominance of bifidobacteria, facultative bacteria are not suppressed and obligate anaerobes such as *Clostridium* spp and *Bacteroidets* spp. are common (Roberts et al., 1992).

Few studies have investigated the composition of the intestinal microbiota at the level of the species and when possible of the strains. In one of these studies *B. breve* is reported as dominant in breast-fed babies (Mitsuoka et al., 1977) whereas *B. longum* and *B. adolescentis,* which are

dominant in adults, are dominant in battle-fed infants (Mevissen –Verhage et al.,1987). It is very important to underline that the incidence of *Clostridium difficile* is high in formula-fed infants (about 50% of the total species)compared to the breast-fed ones(6-20%). Enterobacteria are more common in formula-fed than in breast-fed (Orskow et al., 1975) and staphylococci have been found more frequently in breast fed infants, compared to bottled-fed infants (George et al., 1996, Kay et al., 1990)

Assumption of antibiotics has a negative effect on the commensal microbiota of the newborn causing a severe increase of the enterococci and a substantial decrease of the bifidobacteria (Fouhy et al.,2012).

1.4 The host-microbial interactions and the immune system development

The intestinal microbiota is strictly in contact with the mucosa and with the epithelial cells of the intestine. This human body site is the most extended surface of the body (250-400m^2) after the respiratory apparatus.

To colonize this site, the microorganisms have to develop and to adhere to the mucosa. Adhesion is possible thanks to the specific surface components (proteins and fimbriae) and thanks to competition mechanisms consisting in enzymes and toxins. Specific sites present environmental conditions more advantageous for the colonization of some microbial groups. The microorganisms tie specific receptors of the host-cells determining the “tropism” (Chisari et al., 1992).

The human intestine is the organ with the higher immune activity of the human body (Vickery et al., 2011). The intestinal lumen is separated by the lamina propria through a thin layer of the epithelium covered by intestinal villi and lymphoid aggregates as Payer patch. The villi have the function to increase the surface available for the nutrients absorption. The lymphoid aggregates monitor the microbial content into the lumen. The epithelial cells have specialized functions based on their localization. Specialized enterocytes adsorb the nutrients by the small intestine and the water by the colon and they have microvilli. The epithelial layer is covered by mucus that is metabolized and digest by the commensal bacteria that adhere strictly to the apical part of the enterocytes through specialized pili inducing a tumble of signal through the cytokine.

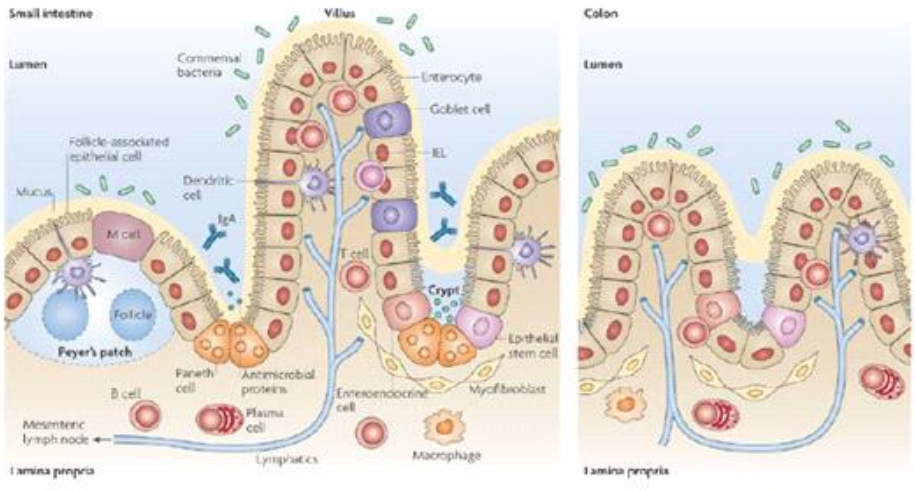


Figure 2: *Interactions between commensal microrganisms and intestinal immune systems (Figure adapted from Abreu, 2010).*

The environment of the intestinal lumen induces the production of polysaccharides and microbial factors by commensal bacteria. Among them are 12cological12i, that promote the exclusion of other microorganisms.

The specialized epithelial cells as M cells (that are presents into the epithelium associated to the follicle) explore the content of the intestinal lumen and start the process of the immune response presenting the antigens to the macrophages and dendritic cells (DC). These cells and macrophages induce the response by the T and B cells. The special polysaccharide coating of commensal microrganisms beckon the recognition by specific receptors (pattern recognition receptors, PRRs) leading to the homeostatic immune response.

The immune response is regulated by the induction of T_{reg} cells and by the differentiation of the plasmatic cells. These last cells secrete the dimeric antibody IgA into the lumen (that bound the antigen and the bacteria) allowing the development of an immune response maintaining the intestinal homeostasis between host and commensal microbiota (Ventura et al., 2012).

The intestinal microbiota plays an important role in the first develop of the immune system in infants. It is a source of microbial stimulation and gives the first signal to drive the post-natal maturation. An inadequate composition of the microbiota during the infancy leads at an immaturity of the intestinal wall that negatively affects the equilibrium between Th1 and Th2 immunity, causing an higher predisposition to the allergic disturbs and most probably to the insurgence of colics (Vael C and Desager K., 2009).

Innate immune response against pathogens starts in the recognition of these ones and with the development of the specific response of T and B lymphocytes together. The DC activity is regulated by the microbial products through several receptors, such as toll-like-receptors (TLR), that produce different responses based on the type on the antigen presented, and the DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3 –grabbing non integrin). The former activates the transcriptional system IkB/NF-kB that produces pro-inflammatory cytokines and co-stimulator molecules, defending the host by the pathogen microrganisms (Di Mauro et al., 2013).

Since the innate immune systems leads to the production of a high number of pro-inflammatory cytokines, it has developed other mechanisms involved in the regulation process including the production of the IL-10. This molecule operates through the activation of the STAT-3 and TGFβ that operates through Smad and MAP.

Another important secretion is that of the IL-6. It is a multifactorial cytokine involved in the numerous biological processes like host response to enteric pathogens, hematopoiesis and acute phase reaction and differentiation of lymphocytes B with pro-inflammatory effects (Montier et al., 2012) .

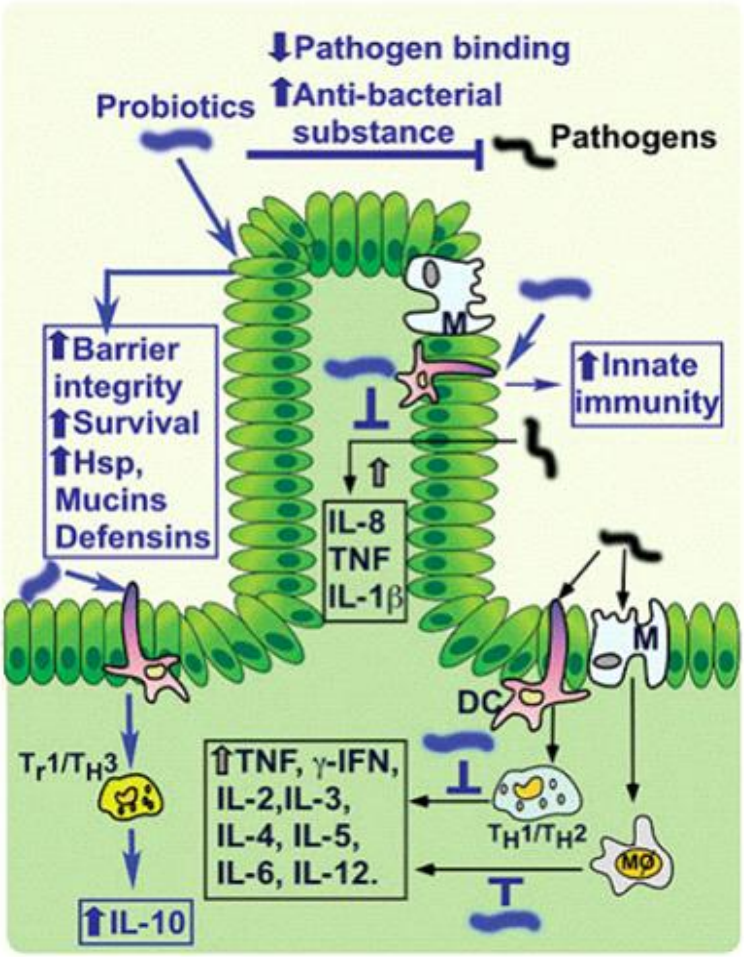


Figure 3: *Innate immune response and cell-mediated immunity (Figure adapted from Vanderpool et al. 2008).*

1.5 How diet influences the intestinal microbiota: saccharolytic and proteolytic metabolism

After weaning, during the first year, the microbiota is characterized by microorganisms capable of degrading more complex sugars and starch, typical of the older infant diet (Backhed, et al., 2015). By 18 months of age, the proportion of organisms that produce short-chain-fatty acids is positively correlated with an increase in body mass index (Bergstrom et al., 2014). By 36 months of age, microbiota composition is similar to the adult one (Arrieta et al., 2014; Avershina et al., 2014; Endo et al., 2014).

In adults the microbiota procures energy and nutriment basically by the carbohydrates or protein fermentation. A high concentration of carbohydrates promotes a saccharolytic metabolism, based on the carbohydrates fermentation, whereas a high quantity of proteins favors the bacteria developing with a proteolytic or putrefactive metabolic activity, based on protein fermentation.
The diet influences the preference to the saccharolytic or proteolytic way, in effect it determines a diverse proliferation of the microbial groups where each one has a diverse specialization in the substrates degradation. For example, diets rich in fibers make available at colon level the complex carbohydrates that lend a competitive advantage to the saccharolytic bacteria among which *Bifidobacterium* and *Lactobacillus*. Vice versa, in diets rich in proteins it is observed an higher concentration of *Bacteroidetes* and *Alistipesgenera,* and a decrease of the *Firmicutes* at intestinal level (Claesson et al. 2012).

1.5.1 Saccharolytic metabolism

Daily, 40 g of complex carbohydrates taken with diet and not digested by the enzymes of the small intestine reach the colon. In this body site they are fermented by the microbiota as source of carbon and energy producing an extraordinarily wide range of metabolites.
The most important types of carbohydrates that reach the intestine are starches, non-starch polisaccharides (NSP) that include the structural polysaccharides of plant cell walls, oligosaccharides and some mono- and di- saccharides (Cummings et al. 1991).
In the large intestine, the proximal colon and caecum are the regions in which the carbohydrates degradation and fermentation takes place. The slightly low pH is determined by the metabolism of these substrates. In effect the carbohydrates fermentation leads to the production of short chain fatty acids (SCFA) such as as acetate, propionate and butyrate.
Acetate is the most abundant, produced by most enteric bacteria as fermentation product, but it is also produced by acetogenic bacteria, such as *B. hydrogenotrophica* from H_2 and CO_2 from formate via the Wood Ljungdahl pathway. The major part of the colonic bacteria require to yield other products in addition to (or instead of) acetate, including succinate, propionate, butyrate, formate, D-lactate, L-lactate and ethanol.
The relative abundance of these products depend on the microbiota composition and also by the other environmental conditions as pH, hydrogen partial pressure and available substrates (Louis et al., 2014).

Propionate is mostly formed by the succinate pathway by *Bacteroidetes* and by some *Firmicutes* or by the acrylate pathway or propanediol pathway. The proportion of the propionate that is present in total fecal SCFA correlates with the relative abundance of *Bacteroidetes* which confirms that succinate pathways is the dominant source of propionate.

Butyrate is produced by some *Firmicutes* using either the butyryl-CoA: acetate CoA transferase route including several of the most abundant species in healthy gut microbiota (including *Facalibacterium prausnitzii*, *Roseburia* spp, *Eubacterium rectale*, *Eubacterium halii* and *Anaerostipes*).

All these three SCFA are rapidly adsorbed from the gut lumen, but their subsequent distribution is different. Butyrate is used as energy source by gut epithelial cells. Propionate is mostly metabolized at liver level and only acetate archives relatively high concentration ranging between 0.10 and 0.15 mM in peripheral blood.

The intracellular propionate and butyrate influence the down-regulation of pro-inflammatory cytokines such as IL-6 and IL-12 in colonic macrophages. SCFA exert potentially important anti-inflammatory effects and have been shown to regulate colonic regulatory T cells (T_{reg} cells) in mice (Smith et al., 2013, Chung et al., 2013). This anti-inflammatory effect is important also for the homeostasis of the gut microbiota. An intriguing hypothesis proposes that anti-inflammatory effect of high butyrate levels tends to limit the immune response toward the gut microbiota, whereas low butyrate concentration trigger a pro-inflammatory state that results in remodeling of the gut microbiota via the suppression of potential pathogens and restoration of butyrate producing species (Chung et al., 2014).

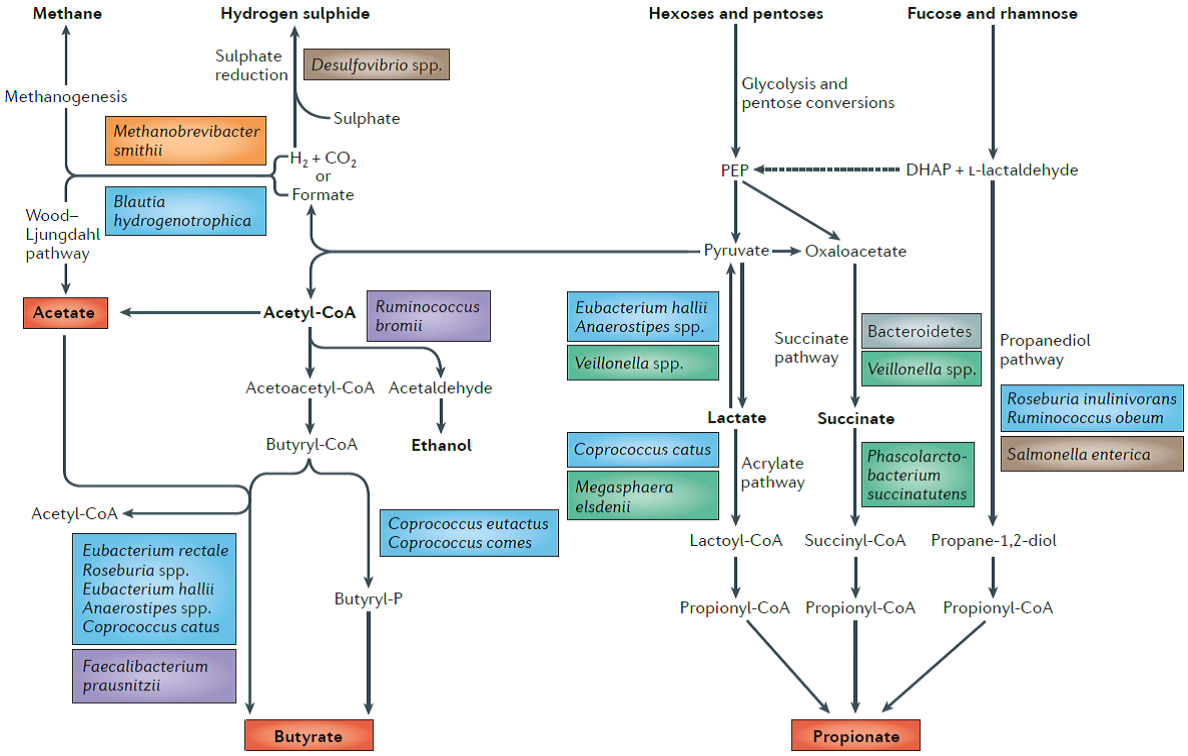


***Figure 4**: Principal pathways and microbial metabolites in carbohydrates fermentation and in the microbial cross feeding. By the three short chain fatty acids (red boxes), acetate could be produced by several enteric microorganisms starting from pyruvate through the acetyl-CoA pathway or through the Wood-L Ljungdahl pathway. Butyrate is formed by the two molecules of Acetil-CoA and it is produced by several Firmicutes. The main pathway of propionate producing way is the succinate pathway that is utilized by the bacteria belonging to the Bacteroidetes in order to make this SCFA starting from carbohydrates. Moreover, some bacteria belonging to Firmicutes phylum could produce propionate by lactate or by succinate. Archeae are highlited in orange, Bacteroidetes in grey, Lachnospiraceae (Firmicutes) in blu, Ruminococcaceae (Firmicutes) in violet, Negativicutes (Firmicutes) in green and the Proteobacteria are highlighted in brown (DHAP, dihydroxyacetonephospate; PEP, phosphoenolpyruvate) (Figure adapted from Louis et al., 2014)*

In the last years have been recognized some interactions within some SCFA (first of all propionate) and the receptors FFAR2 and FFAR3. These receptors could influence the fullness sensation and give a new target for the treatment of pathology as obesity or diabetes increasing the sensibility to the insulin of the patient. (Arora et al. 2011).

1.5.2 Proteolytic metabolism

Every day 12-18 g of not completely digested proteins reach the distal colon. These components came from proteins ingested with diet, but also endogenous protein composed by digestive enzymes and peptides that derived from the renewal of the intestinal epithelial tissue are present. The proteolytic degradation appears in the distal colon where the carbohydrates are less available and the pH is almost neutral (Hamer et al., 2012).

This environment allows the proliferation and surviving of the bacterial groups that glean energy by the amino acids fermentation and contribute to a neutron-basic pH. On the base of the experiments carried out on single cultivable species, it is known that proteins are fermented by the bacteria belonging to the *Bacteroides*, *Clostridium* and *Streptococcus* genera that include species as *Bacteroides thetaiotaomicron, Bacteroides eggerthii, Bacteroides ovatus, Bacteroides fragilis, Parabacteroides distasonis, Clostridium bartlettii* and *Eubacterium hallii*. A diverse system of the chemical reactions as transamination, oxidations and reductions are involved in the disruption and amino acids fermentation in the distal colon where compounds as cheto-acids, H_2, unsaturated fatty acids and other amino acids play the role of electron donors and acceptors to simplify the fermentation process (Fig. 5).

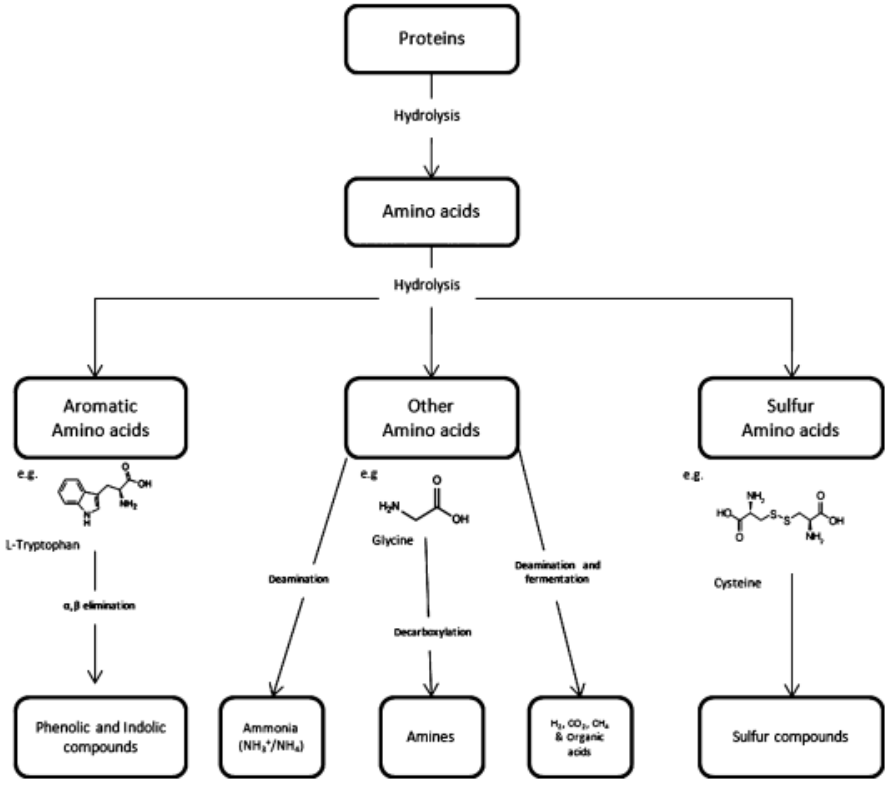


Figure 5: *Pathways of the aminoacids fermentation and final metabolites*

The amino acids structure disruption as valine, isoleucine and leucine leads to the formation of SCFA as acetate, propionate, butyrate and branched chain fatty acids (BCFA) as isobutyrate, 2-methylbutyrate and isolavalerate. This process is carried out mostly by the bacteria belonging to the *Clostridium* genus through the Stickland reaction (Larsson et al 2006). NH_3 is produced by bacteria through the deamination of amino acids and to a lesser extent through urea hydrolysis catalyzed by bacterial urease activity (McIntyre et al., 1993). Up to 3.5-4.0 g of NH_3 is released every day in the gut (Silvester et al., 1997) resulting in luminal concentration in humans up to 60 mmol/Kg of luminal content (McCance and Widdowson 2002). Ammonia alters the morphology of the intestinal tissue and may act as a tumor promoter in the gut.

The fermentation of the aromatic amino acids as phenylalanine, tyrosine and tryptophan leads to the formation of phenolic compounds as indole, p-cresol, and skatole with high potential of toxicity. Normally these metabolites are detoxified and expelled but they became toxic when are accumulated into the tissue because of a not adequate functionality of kidney and liver.

Amines derive by the decarboxylation of the lysine and ornithine which lead to the production of cadaverine and putrescine polyamines. The disruption of these molecules leads to the formation of simplex amines that are adsorbed at colon level. These ones are responsible of the uncontrollable proliferation cellular with potential cancerogenic effect. Indeed, several pathogens including *Shigella flexyneri*, *Streprococcus pneumonie*, *Salmonella entrica* subsp *enterica* serovar, *Typhimurium* and *Helicobacter pylori* exploit polyamines to increase the virulence (Di Martino et al., 2013).

Phenolic compounds as phenyl-acetic-acid, phenol and p-cresol are produced by the partial disruption of the amino acids as tyrosine and phenylalanine by the anaerobic bacteria present into the intestine (*Bacteroides*, *Lactobacillus*, *Enterobacter*, *Bifidobacterium, Clostridium*) (de Loor et al. 2005). These compounds inhibit the endothelial proliferation and reduce the ability of the regeneration of the epithelium (Dou et al. 2004). *In vitro* experiments suggest that p-cresol could have a pivotal role in the dysfunction of the smooth musculature of the vascular endothelial in patient affected by CKD. Moreover, the p-cresylsulfate, but not the p-cresol, presents *in vitro* a pro-inflammatory effect on the leucocyte (Schepers et al. 2007).

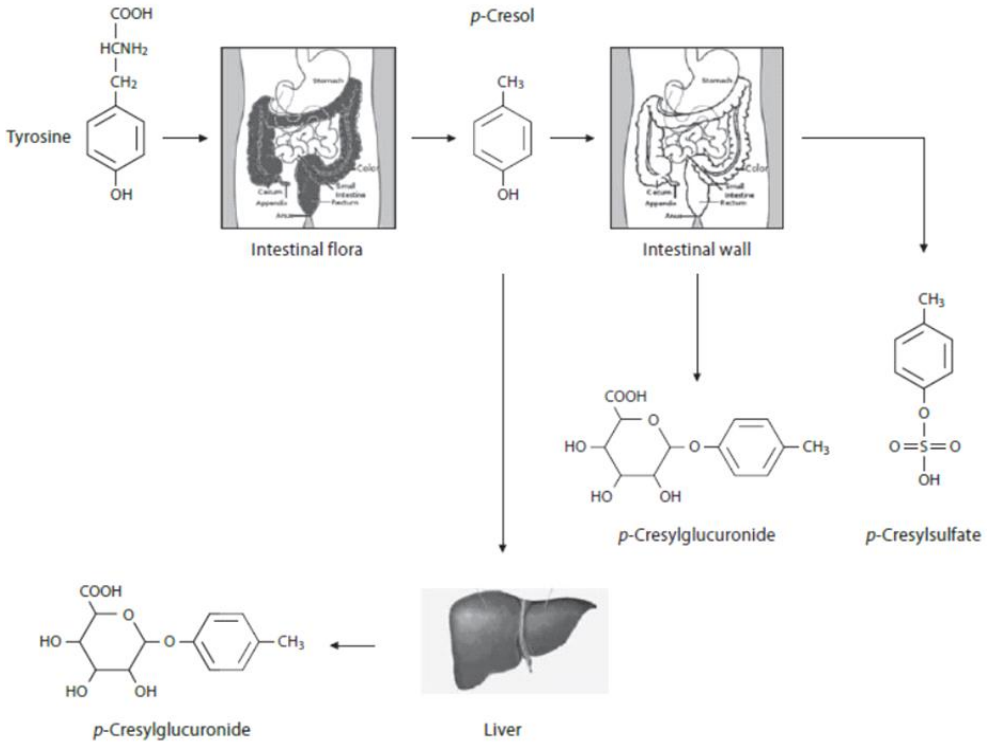


Figure 6: *p-cresol formation starting from tyrosine precursor. At intestinal and liver level the p-cresol is transformed in p-cresylsuphate and p-cresyl-glucuronyde.*

The indolic compounds come from the bacterial metabolism of the tryptophan into the colon. This reaction is carried out by the triptophanases enzymes by the intestinal bacteria such as *E. coli*. Indole is absorbed into the blood from intestine and it is metabolized into indoxylsulfate at liver level (Cummings et al. 1983), while indoxyl sulfate is normally excreted into the urine.

This compound with its precursors as indole leads to the increase of the glomerular sclerosis and to the decrease of the renal functionality. Moreover, the high concentration of this compounds stimulate the transcription of the TGF-β1gene that cause nephrotoxicity and leads to the renal fibrosis (Adijiang et al. 2008).

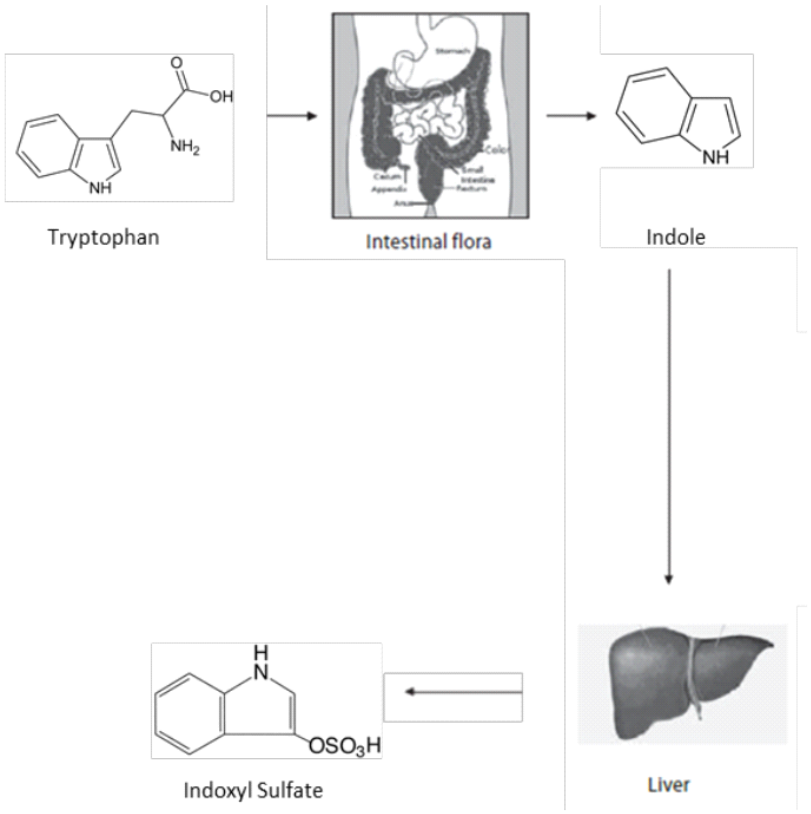


Figure 7: *Indole formation starting from tryptophan precursor. At liver level this compound is metabolized in indoxyl-sulphate.*

Finally, some nitrogenous compounds particularly N-nitroso compounds (NOCs) have the potential to promote cancer and exert carcinogenic effect via DNA alkylation, which can cause mutation (Gill et al., 2002).

All these compounds are called URMs (Uremic Retention Molecules) or uremic toxins. The accumulation over time of these molecules leads to the appearance of several diseases as Uremic syndrome (Matsuki et al., 2013) or cancer or hepatic encephalopathy.

1.6 Probiotics and Prebiotics

The perspective of modulating the composition and metabolism of the gut microbiota using prebiotics, probiotics and synbiotics had been evaluated in several studies. Liu et al 2004 suggested that the modulation of gut microbiota may reduce the total ammonia in portal blood by decreasing bacterial urease activity, decreasing ammonia adsorption by decreasing pH, decreasing intestinal permeability and by improving nutritional status of the gut epithelium (Redha 2013). Moreover,

probiotics and prebiotics may decrease endotoxemia and pro-inflammatory milieu resulting in improvement in inflammation, oxidative stress and liver disease severity and they may decrease the uptake of other toxins.

1.6.1 Probiotics

The term probiotic means “for life” and today is employed to indicate bacteria assumed live that are associated to beneficial effects for humans or animals improving the balance and stability of the intestinal microbiota (Tannock 2002). In the 2002 the FAO/WHO has defined the probiotics as “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host” (Schlundt and Jorgen, 2012).

Microorganisms belonging to different genera are normally used as probiotics in particular strains belonging to the heterogeneous group of bifidobacteria and lactic acid bacteria (LAB). These microorganisms have to satisfy some requirements to be considered probiotics:

- being safe for humans (e.g. according to EFSA or FDA regulation) and lacking antibiotic resistance genes on element;

- being active and vital in the GIT, surviving to the gastric juice, bile salts and to the intestinal content flux reaching the colon (Vasiljevic and Salah 2008);

- being able to live and multiply themselves in the colon (Gorbach 2002);

- being able to confer physiologic benefits.

The effect that the probiotics have in the GIT could be resumed in 3 mechanism of action (Fuller, 1989):

- decreasing of the pathogenic microorganisms in the gastrointestinal tract through the production of antimicrobial substances (lactic acid, acetic acid, acetaldehyde, hydrogen peroxide, carbon dioxide and bacteriocins), the competition for nutrients, the competition for the adhesion to the gastrointestinal epithelial receptors.

- alteration of the microbial metabolism in the GIT through the increase of the activity of specific enzymes as β-galattosidase (it is able to alleviate the difficulty of digestion of lactose) and

the decreasing of the activity of others as nitroreductases in the colon (with procancerogenic effects).

- stimulation of the immune processes.

The bacteria that inhabiting the intestine could be divided into 3 general classes:

- organisms almost always present in high amounts, that colonize a certain district early in life and in this site are permanent residences, the so-called native,

- organism normally present but in relatively limited amount in the resident microbiota,

- organism present in limited number also called allochthonous, probably ingested through diet or coming from other body sites of by the environment.

Bifidobacteria belong to the first class, since they are Gram positive at high G+C content belonging to the *Actinobacteriaceae* phylum and to the family of the *Bifidobacteriaceae* (Ventura et al. 2004). They are non-spore-forming microorganisms, motionless and strictly anaerobic. Bifidobacteria gain energy through a distinctive pathway of carbohydrates fermentation, which yields acetate and lactate as main products, respectively in a ratio of 1.5:1, and does not produce gas. Bifidobacteria are classified by the Food and Drug Administration (FDA) as Generally Regarded As Safe (GRAS) and they are normally employed as probiotics in the formulation of functional foods since their presence into the GIT is associated to important benefits for the human health.

From an ecological point of view, the simple sugars do not represent the carbon source normally utilized by the bifidobacteria in their normal ecosystem. The colon is an organ normally lacking monosaccharides, because of their absorption in the small intestine. These bacteria, as well as others saccharolytic intestinal bacteria, have evolved according to the use of complex carbohydrates that escape the digestion and absorption by the host, developing the ability to use as sources of carbon fibers of food origin and glycoproteins, mucins and glyco-conjugates of human origin (Derensy-Dron et al. 1999, Murphy 2001).

Oligo- and polysaccharides have to be hydrolyzed, in order to enable bacterial fermentation. If the hydrolysis takes place within the bacterial cell, adequate transporters are required to internalize the oligosaccharides. If hydrolysis is performed by extracellular enzymes, it will be followed by the uptake of the saccharidic units released. In this case, the extracellular glycosil-hydrolases could

be produced by the cell itself, and also by other intestinal microbial groups, thus representing an important element of nutritional interdependence among the microbial groups.

With regard to the nitrogen source, several species are able to utilize ammonium salts (*B. breve*, *B. infantis*, *B. longum* and others), while others need of organic nitrogen compounds (Matteuzzi et al., 1978). For some species an important characteristic is the ability to produce amino acids and vitamins, in particular belonging to B vitamins group as thiamine (B1), nicotinic acid (B9) and folate (Deguchi et al. 1985; Pompei et al. 2007). The optimal conditions for the growth are temperatures comprises between 36 and 38 °C, the optimal pH is 6-7. The strictly anaerobiosis is necessary for the *Bifidobacterium* growth.

The *Lactobacillus* genus is the larger of the LAB family. It is very heterogeneous for what concerns the morphologic, physiologic and functional characteristics but it is characterized by a common ability to convert the fermentable carbohydrates prevalently into lactic acid (Makarova and Koonin 2007). The morphology is variable: some species appear as long and thin stick others are shorter and thick. Within each species the length is variable base on the growth phase, the medium composition and oxygen concentration. Lactobacilli are demanding bacteria for a nutritional point of view requiring substrates containing complex nitrogen sources. They present auxotrophies for numerous aminoacids, nucleotides and vitamins since they are adapted in a diverse habitats that may be rich by nutritional point of view (Kandler et al,1986).

Lactobacilli are bacteria able to colonize different ecological niches including plants and food matrices and diverse districts of human or animal body. They could grow in a range of temperature between 5 up to 53 °C with optimal values between 37-40°C. They are are able to grow at pH<5 while the optimal pH is between 5.5 and 5.8.

The *Lactobacillus* species able to colonize the colon in humans have captured the attention of the researchers since they are able to have positive effect on the host health (Heiligh et al., 2002). For this reason, several probiotic formulates for pharmaceutical or food utilization, have been developed with lactobacilli belonging to the following species: *L. acidophilus*, *L. brevis*, *L. casei*, *L. cellobiosus*, *L. crispatus*, *L. curvatus*, *L. debruckii* subsp. *bulgaricus*, *L. fermentum*, *L. gasseri*, *L. 24cologica*, *L.plantarum*, *L. ruteri*, *L. rhamnosus*, *L. salivarius*.

1.6.2 Prebiotics

Prebiotics are indigestible food ingredients that selectively stimulate the growth and the activity of a limited number of species in the colon with a consequent benefits for the host (Gibson et al., 2004; Ouwehand et al., 2006).

Most prebiotics consist of carbohydrates, especially oligo- and polysaccharides, but this definition does not exclude other molecules that could be employed with the same aim. The major part of the prebiotics come from vegetables, while others are produced by the enzymatic way through the hydrolyses of complex polysaccharides or by the transglycosylation of mono and di-saccharides. Prebiotic carbohydrates are not hydrolyzed by the hydrolytic enzymes of the host and they are carbon source for the saccharolytic microbiota of the colon.

The prebiotics allow the increasing not only of the beneficial bacteria but also of their metabolic activity through the availability of the fermentable substrates. This increased metabolism is the key of the prebiotic effect on the host health. Prebiotic fermentation leads to the formation of SCFA (Short chain fatty acids) which are beneficial to the host because they provide energy for the host epithelial cells (Hamer et al., 2008), inhibit pathogen growth (Tuohy et al., 2005) and increase mineral adsorption (Weawer et al., 2011). The polymerization degree and ramification and the type of the glyosidic bonds between the monosaccharides units (xylose, galactose, glucose and fructose) are responsible of the specificity in the use of the prebiotic by the intestinal microbiota. Prebiotics have shown to reduce the production of putrefactive compounds (Rowland 1995). In effect, the principal aim of the prebiotics assumption is the increasing of the number and activity of the bifidobacteria and lactic acid bacteria present in the colon, with a reduction of the putrefactive microorganisms or potential pathogens like some clostridia and enterobacteria (Campbel et al., 1997; Rycroft et al., 2001).

In effect in the study of Maathuis et al., 2012 the authors have shown that *B. bifidum*, *B. catenulatum*, *L. gasseri* and *L salivarius* especially fermented the galacto-oligosaccharides. Moreover, their study has revealed that microorganisms as *Klebsiella* and *Enterobacteriaceae* increase they their concentration too but this increase is low compared to that of beneficial bifidobacteria and lactobacilli.

The target of the action of the prebiotic is the intestinal ecosystem but indirectly is the entire organism. In order to reach this aim is possible to assume not only probiotics or prebiotics but also symbiotics that are constituted by a specific mix of both these components.

Examples of prebiotics are galacto-oligoisaccharides and fructo-oligosaccharides and inulin.

In the last years the first two one are frequently use in the preparation of formula-milk (Ninonuevo MR and Bode L, 2008).

In effect, some companies started to supplement their formulas with oligosaccharides that are structurally different, but show similar prebiotic and immunomodulatory effects.

GOS/FOS supplemented formulae have also been reported to modulate the immune system in mice (Vos et al ., 2006) and to reduce the incidence for infectious episodes (Arslanoglu et al., 2007) and atopic dermatitis in at risk infants (Moro et al., 2006).

Galacto-oligisaccharides (GOS) are oligomers of galactose units bound to a terminal glucose. The monomeric units are bound by glyosidic bonds β-(1→4). Into this family the lighter is lactose, it is a disaccharide of glucose and galactose. GOS are normally present in the human milk and they could be produced starting from lactose through the β-galattosidase (β-Gal) enzyme. This enzyme in an hydrolase that hydrolyzes the β-(1→4) galactosides transferring the galactose by lactose in the water. The enzymatic reaction leads to a blend of oligosaccharides, also called transgalacto-oligosaccharides (TOS), which present a variable polymerization degree between 3 and 6 with a mean value of 3-4 monomeric units. TOS are not hydrolyzed or adsorbed in the small intestine and has been observed that they are rapidly fermented in the proximal colon. In particular, they are used by the bifidobacteria and it was underlined that their use leads to the proliferation *in vivo* of this specific genus (Varnazza et al., 2006; Bouhnik et al., 1997).

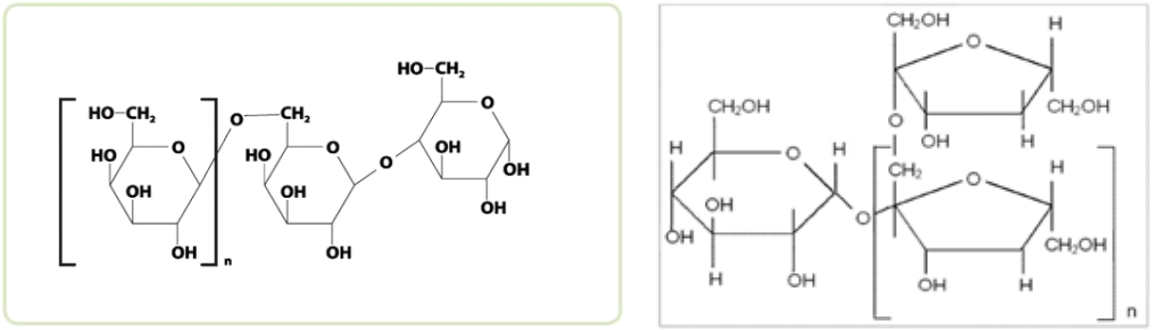


***Figure 8**: Structure of GOS (left) and FOS (right)*

Fructo-oligosaccharydes are natural ingredients normally present as carbohydrates reserve in several vegetables and plants. They are carbohydrates characterized by a variable number of monomeric unit between 3 up to 10. These are industrially made through 2 principal methods that differs for the composition of the final product. They could be produced starting from saccharose through the β-fructosiltransferase enzymes activity or through the enzymatic hydrolyses controlled of the polysaccharides inulin. The reaction on the base of the synthesis of the FOS requires high concentration of substrate (saccharose) in order to occur correctly and efficiently allowing to obtain FOS characterized by 2-4 units connected by β(1→2) bonds unit to a terminal of α-D-glucose

The evidence that the assumption of FOS as prebiotic decreases the concentration of microorganisms belonging to *Bacteroides* and *Clostridium* have been demonstrated. The increase of the concentration and proliferation of the bifidobacetria is followed by other positive effects related to the FOS introduction like the modulation of the intestinal functionality, the decrease of the fecal pH, the modulation of the cholesterol level and a better adsorption of calcium and magnesium (Roberfroid 2005). The carbohydrates fermentation is important for the micro ecology and physiology of the large intestine because it generates lactic acids and short chain fatty acids that are absorbed by the host or utilized by other microorganism (Cummings and MacFarlane 1991; Hoverstas et al., 1984) creating the opportunity not only for the competition but also for the cooperation through the metabolic cross-feeding.

Recently it has been demonstrated the important effect of prebiotic mixture for the infants composed by 90% of galacto-oligosaccharydes and 10% fructo-oligosaccharides. This composition has been chosen in order to obtain a prebiotic effect similar to that of the human milk. In effect, it favors the proliferation of bifidobacteria and lactobacilli that became comparable to that present in the feces of infants breast-fed. *In vitro*, the short chain fatty acids produced by the mixture of oligosaccharides are similar to that produced by the human milk, but they differ significantly by that of the infants formula fed without supplements (Bohem et al., 2004). Moreover, the fecal pH seems higher in infants fed with formula milk without the adding of prebiotics than of the newborn breast or formula fed integrated with a prebiotic blend (Balmer et al., 1989).

2. Aims

This theses project faced diverse aspects of the relationship between the composition of the intestinal gut microbiota, its metabolism, and the effects on the health.

A first study focused on the *in vitro* validation of a the possible intervention aimed to alleviate colics, an infant disease correleated to high abundance of *Enterobacteriaceae* in the gut microbiota. This approach is based on the supplementation of a I probiotic strain of *Bifidobacterium*. The rationale of the study rests on the consolidated utilization of probiotic lactobacilli for the treatment of infant colics and on a previous screening which yielded a strain of *B. breve* B632, capable of inhibiting the growth of coliforms isolated from colic newborns. With this purpose, a continuous bioreactor process was developed for cultivation of the microbiota from a colicky infant. The cultures were supplemented with the potential probiotic, then the impact on microbiota composition was evaluated using culture-independent techniques for the quantification of *Enterobacteriaceae*.

A further study aimed to investigate protein fermentation by the gut microbiota. Bacterial protein catabolism has relevant effects on health, since aminoacid fermentation yields a number of toxic compunds (e.g. indole, *p*-cresol, ammonia) which are absorbed in the colon and enter into the bloodstream. In particular, bacterial protein fermentation occurring in the colon has a major contribution to the levels of the so-called uremic toxins and thus is particularly relevant for the health status of subjects with compromised renal function. In the present study the composition of the bacterial population of the gut microbiota involved in protein fermentation was investigated. For this purpose, a medium containing proteins and peptones as the sole fermentable carbon source was developed and utilized in bioreactor experiments to enrich the gut microbiota with the bacteria gaining energy from protein breakdown. Metagenomic 16S sequencing of samples from microbiota cultures was utilized to identify, at the deepest possible taxonomic level, the bacterial taxa which throve in enrichment cultures and are possibly involved in protein fermentation and in generation of uremic toxins *in vivo*.

Furthermore, a cohort of patients with chronic kidney disease and one of heathy subjects were enrolled. The feces of the cohorts were compared, in order to point out the difference in terms of the chemical composition and bacterial metabolism, with respect to the generation of indole and p-cresol. Chemical characterization included the profile of volatile organic compounds and the main uremic toxins that could be generated by gut bacteria. Such a comparison will be contextualized in a metagenome-based survey of the microbiota composition of the two cohorts.

As a whole, these three main topics share the functional study of gut microbiota and possible strategies to modulate it, in order to maintain the health status, or to prevent or alleviate microbiota associated diseases.

3. Investigation on the *Bifidobacterium breve* B632 ability to inhibit the growth of *Enterobacteriaceae* population in a model system of intestinal microbiota

3.1 Background

In the first part of the thesis project the ability of the *Bifidobacterium breve* B632 strain to inhibit the growth of the *Enterobacteriaceae* population, the main responsible of the insurgence of the colics symptoms, was investigated.

The infantile colic is a gastroenteric disorder that is generally common in newborns with age lower than 4-5 months without a clear organic base. In general, the term "colic" implicate an abdominal pain secondary to an obstruction of the blood flow by an organ like kidney, gallbladder or intestine. In particular, it is behavioral, and characterized by a paroxysmal crying, flushed face, cries for more than 3 hours a day, for at least 3 days a week for more than one week, without any evidences able to clarify the origin of the disturb by gastroenteric organ or by other body sites (Wessel et al., 1954).

Its etiology is not well understood. The immaturity of the newborn nervous system, the mother-son interaction, the intestinal motility disturb, the hormonal intestinal alterations as motilin and grelin could be considered the casual factors predisposing for the syndrome developing (Rhoads et al., 2009). Recent studies have underlined an important role exercised by the intestinal microbiota that are associated to a diverse metabolism of the carbohydrates and fatty acids (Lehtonen et al., 1994).

As reported before, studies performed on the classical methods and molecular analysis underlined important differences between the microbiota of the health newborns and infants with colics. The most recent study (de Weerth et al., 2013) based on molecular techniques that analyze quali- and quantitatively the entire microbiota, has underlined that in general the newborns with colics present a minor diversity of the microbiota and a lower stability over time. This minor diversity indicates that the intestinal microbiota of the colicky infants diversifies more slowly.

According to the studies conducted with traditional techniques, the proteobacteria are two times more concentrated in infants with colic compared to the control ones. Bacteria belonging to genera *Escherichia*, *Klebsiella*, *Serratia*, *Vibro*, *Yersinia* and *Pseudomanas* genera are particularly abundant. It is well known that *Escherichia* and *Klebsiella* species are gas producing from their fermentative metabolism and they produce proinflammatory lipopolisaccharides. For this reasons these bacteria could contribute to the pathology with both these mechanisms. On the contrary, the *Bacteroidetes* and *Firmicutes* are less represented (de Weerth et al., 2012; Ross et al., 2013).

The analysis of cultivable microorganisms have underlined a lower population of lactobacilli and an higher number of the Gram-negative bacteria (de Weerth et al., 2013). In particular, relatively to the lactobacilli, the study of Savino et al., showed that colicky infants have an altered

microbiota, deficient in lactobacilli; this could leads to the immaturity of the gut barrier and to aberrant antigen transfer and immune response, thus explaining increased vulnerability to the breaking down of oral tolerance, as reported by different studies (Kalliomäki et al., 2001, Iacono et al., 1991, Lothe et al., 1990). In effect several studies highlighted that lactobacilli have an important role in the intestinal immune system development: they can induce the production of γ-iterferon, which is important in the reduction of Th2 cytokines and in the building of a correct intestinal barrier. The group of *L. plantarum* seems less represented (it comprises *L. rhamnosus*). Based on the studies conducted on the analysis of the cultivable microbiota, heterofermentative lactobacilli (*L. brevis and L.lactis*), producing ethanol and CO_2 in addition to lactic acid, have been reported only in infants with colics, while homofermentative species (*L. acidophilus*) have been identified in healthy ones (Savino et al., 2005; Savino et al., 2004). The increasing of meteorism and flatulence, that are typical of this disease, may be caused by these differences. Moreover methane production is lower in the fecal samples of newborns with colics, suggesting an inability of the intestinal microbiota to convert the hydrogen in methane with a consequent accumulation of the gastrointestinal hydrogen (Belson et al., 2003).

The assumption of probiotic bacteria (*Bifidobcterium* spp and *Lactobacilli* spp) during the infancy and the interactions that they could explicate with the intestinal cells play an pivotal role in the activation or modification of the innate immune response (Vinderola et al., 2005). Recent studies have underlined the ability of these microorganisms to induce the production of IL-6 by the epithelial cells (Nissen et al., 2009). It have been also demonstrated that the LAB bacteria and bifidobacteria are able to use the TLR receptors to send signals of immune response to the cells taking part in the development of the immune response in the first months of life. A qualitative or quantitative alterations could be involved in the infantile colics insurgence.

In the first month of 2002 the Food and Drug Administration has accepted the use of *Bifidobacterium lactis* in the formula milk conferring the state of Generally regarded as safe (GRAS) (Hammerman et al., 2006). Moreover, it was observed that early in life the subministration of *Bifidobacterium breve* promotes the bifidobateria colonization and the consolidation of a normal intestinal microbiota (Li et al. 2004).

Based on these studies, with the aim to perform specific probiotics for the control of the colics, it has been determined the capability to inhibit the growth of pathogenic bacteria by the collection of bifidobacteria isolated by the microbiota of infants *in vitro*. It was observed that some strains are able to contrast the bacterial growth principal responsible of diarrhea of microbial origin in infants (*E.coli*, *Salmonella enteriditis*, *Clostridium difficile* and *Campylobacter jejuni*) (Rowland et al. 2008; Van niel et al.2002).

In particular, the *Bifidobacterium breve* B632 strain, that is the object of the first part of this thesis project, exhibited an antimicrobial activity against the coliforms gas producers isolated from the feces of colicky infants (Aloisio et al., 2012). This strain was subject to deep investigation, in order to accept it as a potential probiotic. It was excluded any possibility to transfer antibiotic resistances to pathogen bacteria. Moreover, the exposition of the strain to H4 and TLT cells determined a significantly increase of the IL6 production, a probably indicator of the macrophages presence and the activation of the endothelial cells and consequently a probably activation of the positive immune response by the host. In conclusion this study indicated *B. breve* B 632 (DSM 247006) as a safe and promising probiotic for the treatment of the enteric disorders in newborns.

In order to test *in vitro* the abilities of this potential probiotic strain, a model system of infant intestinal microbiota has been developed.

The experiment was structured as reported below:

- set-up and optimization of a continuous fermentative process in bioreactor able to mimic the real composition of the infant intestinal microbiota for the study of colonization by the probiotic strain *Bifidobacterium breve* B632,
- study of the ability of *B. breve* B632 to colonize an exogenous microbiota in the bioreactor,
- study of the *Enterobacteriaceae* decreasing in presence of the *B. breve* B632, through the quantification of the microbial groups of interest,
- quantification of the carbohydrates consumed and the fermentation products generated by the microbiota during the cultivation.

3.2 Materials and Methods

3.2.1 Experimental design

Bifidobacterium breve B632 was obtained from BUSCoB strain collection (Scardovi Collection of Bifidobacteria, Dept. of Agro-Environmental Science and Technology, University of Bologna, Italy). The strain was accepted for deposit by DSMZ for patent purposes and named *Bifidobacterium breve* DSMZ 24706.

Two experiments have been performed. Fresh feces from a 2 months breast-fed colicky infant, born by natural delivery and not treated with antibiotics or probiotics, were utilized to prepare the inoculum for single-stage continuous cultures.

The first set of fermentations, in triplicate, has been realized inoculating in the bioreactor the fecal slurry. These cultures are referred to as microbiota cultures (MC). The second set of fermentations, performed in quadruplicate, was inoculated with the fecal sample and the probiotic strain. These cultures are referred to as probiotic-supplemented microbiota cultures (PMC).

The fermentations were performed in chemostat preparing couples of bioreactor fed in parallel. Each process lasted 24 hours.

During the fermentation samples of 15 mL were collected every 6 hours, in order to monitor the microbial composition and the trend of the substrates an metabolites produced.

3.2.2 Medium Composition

The cultures of gut microbiota were performed in a microbiota medium (MM). This medium was developed on the basis of the medium performed by Macfarlane for the fecal cultures (Macfarlane et al, 1989) and modified by Walker (Walker et al, 2005) considering that the infant has a diet different by the adult one and that the human milk is the only source of nutriment.

The medium is composed by peptones as nitrogen sources and the carbon source was substituted with 6.0 g/L of a mixture of galacto-oligosaccharides (GOS, Domo Vivinal, Needseweg, The Netherlands) and fructo-oligosaccharides (FOS, Beneo-Orafti P95, Oreye, Belgium). The mixture was composed of 90% GOS and 10% FOS (w/w), based on the new formulations of infant formula.

The medium composition is reported below:

- Casein hydrolization 1 g/L,
- Peptone 1 g/L,
- KH_2PO_4 2 g/L,
- NaCl 4,5 g/L,
- $MgSO_4$ $7H_2O$ 0,5 g/L,
- $CaCl_2$ $2H_2O$ 0,045 g/L,
- $FeSO_4$ $7H_2O$ 0,005 g/L,
- Hemin 0,01 g/L,
- Bile salts 0,05 g/L,
- Resazurin 1 g/L 6 mL,
- Antifoam 0,2 mL/L.

2L of medium have been prepared in a tank for the bioreactors feeding. The container and the bioreactors have been sterilized at 121°C for 30 minutes.
After the sterilization the following solutions were added through filtration:

2 mL of mineral solution:

- EDTA 500 mg/L,
- $FeSO_4$ $7H_2O$ 200 mg/L,
- $ZnSO_4$ $7H_2O$ 10 mg/L, $MnCl_2$ $7H_2O$ 3 mg/L,
- H_3BO_3 30 mg/L,
- $CoCl_2$ $6H_20$ 20 mg/L,
- $CuCl_2$ $2H_2O$ 1 mg/L,
- $NaCl_2$ $6H_20$ 2 mg/L,
- $NaMoO_4$ $2H_20$ 3 mg/L;

1.4 mL of vitamine solution:

- K menadione 1 g/L,
- Byotin 2 g/L,
- Pantotenate 2 g/L,
- Nicotinamide 10 g/L,
- B12Vitamin 0,5 g/L,
- Pholate 0,5 g/L,
- Tyamine 4 g/L,
- PABA 5 g/L;

40 mL of reducing solution:

- L-cysteine HCl 0,5 g/L,
- $NaHCO_3$ 3,2 g/L.

3.2.3 Cultures of gut microbiota

The infant feces were homogenized (10%, w/v) in MM with 10% glycerol (v/v), and stored at -80°C until use. The inoculum preparation was performed in anaerobic cabinet under a 85% N_2, 10% CO_2, 5% H_2 atmosphere.

In MC cultures 5 ml of fecal suspension was thawed at 37°C and utilized to inoculate bench-top bioreactors (Sixfors V3.01, Infors, Bottmingen, Swiss) containing 250 mL of MM. The dilution rate was of 0.042 h^{-1}, corresponding to one turnover per day.

The medium was continuously flushed with CO_2 maintaining the anaerobiosis. The temperature was set at 37°C, and it was guarantee a gentle agitation. The pH was maintained at 6.5 through the automatic titration with 4 M NaOH.

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

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

3.2.4 Fluorescent In Situ Hybridization (FISH)

The total bacteria, bifidobacteria, and *Enterobacteriaceae* were enumerated through FISH based on the procedure of Harmsen et al 1999, with slight modifications. Samples were diluted 1:4 with 40 g/L paraformaldehyde, and incubated overnight at 4°C. Fixed cells were washed with PBS at pH 7.4, then dehydrated with PBS-ethanol 1:1 solution for 1 h at 4°C. In order to enumerate the total bacteria, bifidobacteria, and *Enterobacteriaceae* the following probes Eub 338, Bif 164, and EnterobactD respectively were used (Walker et al., 2005).

Eub 358 FITC- GCT GCC TCC CGT AGG AGT

Bif 164 FITC – CAT CCG GCA TTA CCA CCC

EC1531 FITC – CACCGTAGTGCCTCGTCATCA

EnterobactD FITC – TGC TCT CGC GAG GTC GCT CTT

To perform hybridization, 10 µL of cell suspension, 1 µL of the specific FITC-labeled probe, and 100 µL of hybridization buffer (20 mM TRIS-HCl, 0.9 M NaCl, 0.1% SDS) were mixed and incubated for 16 h at the temperature specific for each probe (Walker et al., 2005).

A proper amount of the cell suspension was diluted in 4 mL of washing buffer (20 mM TRIS-HCl, 0.9 M NaCl), and maintained at hybridization temperature for 10 min before being filtered onto 0.2 µm polycarbonate filters (Millipore, Ettenleur, Netherlands). Filters were mounted on microscope slides with Vectashield (Vector Labs, Burlingame, California). The slides were evaluated with a fluorescence microscope (Eclipse 80i, Nikon Instruments) equipped with mercury arc lamp, FITC specific filter, and digital camera. Depending on the number of fluorescent cells, 30 to 100 microscopic fields were counted and averaged in each slide. Each sample was enumerated in triplicate. The image have been acquired through the Nis-element platform.

3.2.5 qPCR

In order to quantify the microbial groups of interest, the biomass from MC and PMC cultures were collected by centrifugation, suspended in PBS (pH 7.8), and extracted with QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) to obtain bacterial gDNA.

gDNA was quantified with NanoPhotometer P-Class (Implen GmbH, Munchen, Germany). It was diluted to 2.5 ng/µL in TE buffer pH 8, and subjected to qPCR analysis. The set of primers Eco-F (GTTAATACCTTTGCTCATTGA) / Eco-R (ACCAGGGTATCAATCCTGTT), and Ent-F (ATGGCTGTCGTCAGCTCGT) / Ent-R (CCTACTTCTTTTGCAACCCACTC) were used for *Enterobacteriaceae* and *Escherichia coli* respectively. (Rintillä et al., 2004, Melinen et al., 2003, Castillo 2006)

The mixture contained 10 µL of SsoFast EvaGreen Supermix, 4 µL of each 2 µM primer, and 2 µL of template. qPCR reactions were carried out with the CFX96 Real-Time System (Bio-Rad Laboratories, Redmond, WA, USA), according to the following protocol: 98°C for 2 min; 45 cycles at 98°C for 0.05 min, 60°C for 0.05 min, and 95°C for 1 min; 65°C for 1 min.

3.2.6 Isolation of bifidobacteria on selective medium

In the PMC fermentations fresh culture samples correspondent to all time points were serially diluted in Wilkins-Chalgren anaerobe broth (Oxoid) in the anaerobic cabinet, and plated on RB selective medium (D(+) Raffinose 7,5 g/L, Sodium acetate 2,5 g/L, Sodium caseynate 5,0 g/L, Yeast extract 5,0 g/L, Lithyum clorure 3,0 g/L, Sodium propionate 15,0 g/L, L-cysteine clorohydrate 0,5 g/L, Sodium thioglycolate 0,5 g/L, Red bromocresol 10,0 ml/L, Salt solution RB 40,0 ml/L).

Plates have been incubated at 37°C in anaerobiosis and after 24-48h the colony forming units have been calculated.

By the plates corresponding to the higher dilutions single colonies have been picked and purified onto MRS medium (DifcoTM Lactobacilli MRS Broth) with cysteine at 0.05%. The genomic DNA was extracted from 200 colonies isolated using Instagene matrix (Bio-Rad).

3.2.7 Taxonomic assignment through PCR genus specific

The genomic extract corresponding to the 200 colonies isolated has been processed using a couple of primers Bif 164 (5' –GGGTGGTAATGCCGGATG –3') and Bif 662 (5' –CCACCGTTACACCGGGAA –3') in order to confirm that all isolates are belonging to *Bifidobacterium* genus.

The components of the PCR mix are: 7,5 µL 2X PCR Master Mix, 1,875µL primer-f (2.5 µM), 1,875 µL primer-r (2,5 µM), 3,75µL template. The final volume of each reaction is 15 µL. The amplification protocol is reported as following: 95°C for 2 minutes;35 cycles at 95°Cfor 45 seconds 55°C for 45 seconds 72°C for 45 seconds; 72°C for 7 minutes.

At the end of the amplification the amplicons were charged on agarose gel 1% in TAE buffer 1X (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) setting the voltage to 160V for 2 hours. The molecular weight marker was GeneRuler DNA Ladder Mix (100-10000 bp). Bands were visualized under ultraviolet light after staining with ethidium bromide, followed by digital image capturing.

3.2.8 RAPD-PCR tracing of *Bifidobacterium breve* B632

In order to trace the potential probiotic strain during the whole process in PMC cultures, the RAPD-PCR was carried out in a 15 µL reaction mixture: 10X Dream Taq Buffer (including MgCl2 2mM), 1.5 µL; dNTPs mixture 0.10 mM, 0,15 µL; 2 µM M13 primer (GAGGGTGGCGGTTCT), 3.75 µL; genomic DNA, 3 µL; PCR water 5.25 µL. DNA amplification was performed with the following protocol: 94°C for 4 min (1 cycle), 94°C for 1 min, 34°C for 1 min, 72°C for 2 min (45 cycles); 72°C for 7 min (1 cycle).

The PCR products were electrophoresed in a 2% agarose gel (25 × 25 cm) for 4 h at a constant voltage (160 V) in TAE buffer.

After staining with ethidium bromide RAPD-PCR profiles were visualized under ultraviolet light, followed by digital image capturing.

The resulting fingerprints were analyzed by the Gene Directory 2.0 (Syngene, UK) software package.

The similarity among digitalized profiles was calculated and a dendrogram was derived with an unweighted pair-group method using arithmetic means (UPGMA).

3.2.9 Analysis of fermentation products

The samples withdrawn at time 0 and after 6, 12, 18 and 24 h of fermentation were clarified through centrifugation (13,000 × g, 5 min, 4°C) and filtration (0.22 µm cellulose acetate filter), and stored at -20°C until analyzed.

Fermentation products (formic, acetic, lactic, propionic, butyric, succinic acids, and ethanol) were analyzed using a HPLC device (Agilent technologies, Waldbronn, Germany) equipped with refractive index detector and Aminex HPX-87 H ion exclusion column. Isocratic elution was carried out with 0.005 M H_2SO_4 at 0.6 mL/min (Amaretti et al ., 2013).

3.2.10 Statistical analysis

All values are means of four separate experiments (però prima diciamo che sono uno in triplicate ed uno ripetuto 4 volte. Comparisons were carried out according to Student's t test. Differences were considered statistically significant for $P < 0.05$.

3.3 Results

3.3.1 Evolution of bacterial groups in probiotic-supplemented and control microbiota cultures

In order to study the potential probiotic activity of the *B. breve* B632 against *Enterobacteriaceae*, single-stage continuous fermentations of the colonic microbiota from a colicky newborn were carried out for 24 h.

Total bacteria, bifidobacteria, *Enterobacteriaceae* and *E. coli* were monitored in both probiotic-supplemented (PMC) and in control microbiota cultures (MC). FISH technique was utilized for total bacteria, bifidobacteria, *Enterobacteriaceae* (Graphic 1A and B), while qPCR was utilized for *Enterobacteriaceae* and *E. coli* (Figure 9).

After 18 h of cultivation, FISH bacterial counts became steady in both MC and PMC cultures. Eubacteria increased up to 9.0–9.4 E+09 cfu/mL, without statistically significant difference between PMC and MC ($P > 0.05$). At all the time-points, bifidobacteria were more abundant in PMC than in MC ($P < 0.05$). *Enterobacteriaceae* were negatively affected by the presence of *B. breve* B632 and were always less numerous in PMC than in MC ($P < 0.05$).

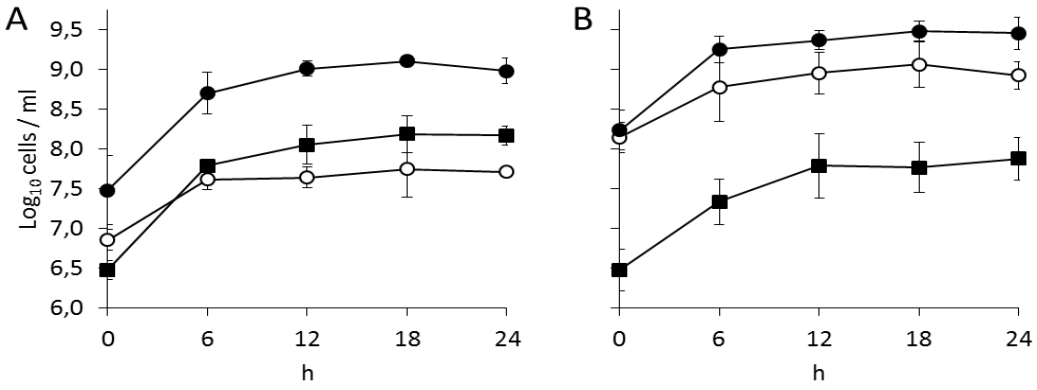


***Figure 9**: Time-course of total bacteria, bifidobacteria, and Enterobacteriaceae in cultures of infant gut microbiota. Eubacteria (●), Bifidobacterium (○), and Enterobacteriaceae (■) were quantified by FISH in control cultures (MC, panel A) and in cultures supplemented with B. breve B632 (PMC, panel B). Data are means ± SD, n = 4.*

Consistently with FISH results, qPCR revealed that *Enterobacteriaceae* were significantly lower in PMC than in MC ($P < 0.05$). On the other hand, statistically significant difference was not observed in the levels of *E. coli* ($P > 0.05$), with the exception of 18 h, when *E. coli* was less numerous in MC than in PMC (Figure 10).

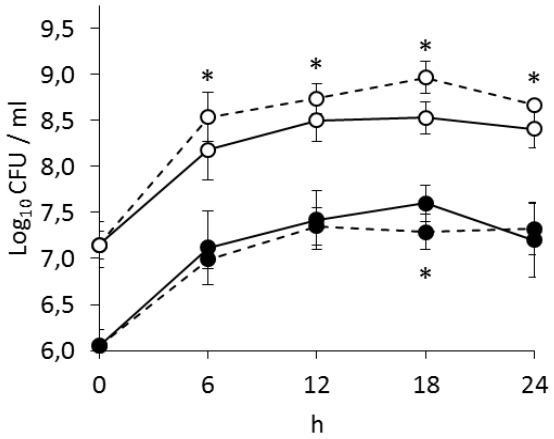


Figure 10: *Time-course of E. coli and Enterobacteriaceae in cultures of infant gut microbiota. E. coli (●) and Enterobacteriaceae (○) were quantified by qPCR in control cultures (MC, dashed line) and in cultures supplemented with B. breve B632 (PMC, solid line). Data are means ± SD, n = 4. Stars indicate statistically significant difference between MC and PMC cultures (P < 0.05).*

3.3.2 Fingerprinting analysis of the isolates

RAPD-PCR fingerprinting was utilized to trace the presence of *B. breve* B632 within bifidobacterial population during incubation of PMC cultures. For this purpose, 200 colonies were isolated onto RB plates at each time-point from PMC cultures. All the colonies were attributed to the genus *Bifidobacterium* by genus specific PCR. At the beginning of the fermentation, the RAPD profile of *B. breve* B632 represented the 85% of bifidobacterial isolates in from PMC cultures, then it decreased to 73% after 6 h and stabilized at 64% at the steady state ($n = 4$, $SD < 34\%$). Considering that at the steady state bifidobacteria accounted for 42cologi. 38% of total eubacteria, according to the FISH enumeration, *B. breve* B632 could be estimated as 42cologi. the 24% of total bacterial population in PMC. In samples from steady state PMC cultures, 2 biotypes of bifidobacteria represented the autoctonous component. The same two biotypes were identified also at the inoculum in MC cultures, together with two other minor ones, none of them exhibiting a RAPD-PCR profile similar to that of *B. breve* B632.

3.3.3 Accumulation of fermentation products

Cultivation of the gut microbiota lead to accumulation of fermentation products. The products of bacterial metabolism in PMC and MC cultures were formate, acetate, lactate, propionate, butyrate, and ethanol (Figure 11 A and B).

In agreement with bacterial counts, the concentrations of the fermentation products became stationary after 43cologi. 18 h. Ethanol, formate, lactate, and acetate were the first to increase at the beginning of the fermentations. Propionate, 2,3-butandiol, and butyrate accumulated later, while lactate decreased as the steady state was approached.

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

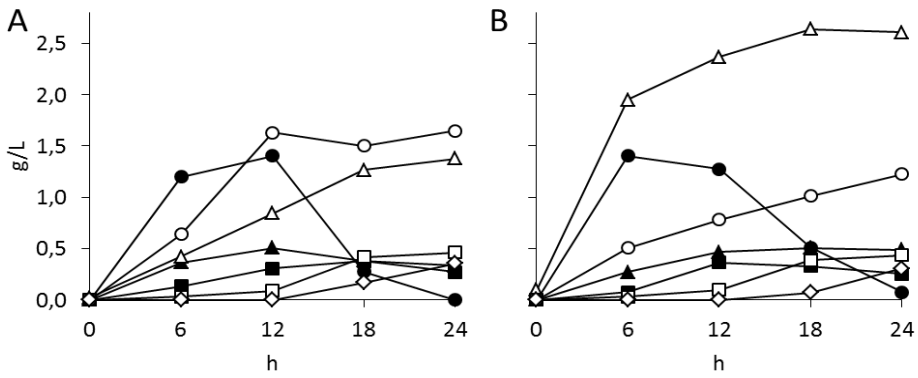


***Figure 11**: Time-course of fermentation products in cultures of infant gut microbiota. Ethanol (○), lactate (●), acetate (△), formate (▲), propionate (□), 2,3-butandiol (■), butyrate (◇) were determined in control cultures (MC, panel A) and in cultures supplemented with B. breve B632 (PMC, panel B). Data are means, n = 4, SD always < 0.25 g/L.*

3.4 Discussion

Unlike strains of *Lactobacillus* spp., scarce information is currently available on the use of bifidobacteria as probiotic for infant colics.

In the study of Di Gioia et al., 2014, *B. breve* B632 was chosen from a panel of *Bifidobacterium* strains as the candidate strain for the treatment of colics. The potential probiotic was selected due to its antimicrobial activity against coliforms, coupled to the lack of transmissible antibiotic resistance traits and cytotoxicity for the gut epithelium. Moreover, the strain is capable of adhering to gut epithelium cell lines and could stimulate gut health by increasing metabolic activity and immune response of epithelial cells (Aloisio et al., 2012).

In the present work the potential probiotic activity of *B. breve* B632 against coliforms was tested in gut microbiota fecal cultures of a newborn of 2 month years old coliky infant, simulating *in vitro* the colonic composition in terms of microbiota and chemical-conditions.

B. breve B632 survived in the model system and persisted in high abundance under steady state conditions. In presence of the probiotic, the family *Enterobacteriaceae* was significantly less numerous at all the time-points compared with control cultures. These results suggest that *B. breve* B632 exerted anti-microbial activity against coliforms in fecal cultures as well, consistently with previous observation with spot agar tests and co-cultures (Aloisio et al., 2012).

However, it seems that *E. coli* counts were not affected by the presence of the probiotic. This observation could be explained by the different specificity of the primer sets utilized in qPCR technique. In effect, the primers for *Enterobacteriaceae* are expected to recognize a broader spectrum of species than the ones for *E. coli* (Table 1).

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

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

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

The high concentration of acetate in PMC cultures is in agreement with the higher abundance of bifidobacteria, compared to MC cultures. In fact acetate is one of the end-products of the genus *Bifidobacterium*, which yields almost equal amounts of lactate and acetate during the fermentation of hexoses (Maathuis et al., 2012). Moreover, the mixture of GOS and FOS utilized in the present study, in the same relative composition (GOS:FOS 9:1, w/w) of the most common prebiotic infant formula, confirmed as potent bifidogenic carbon source.

Systems as the one herein described are currently the best tools to investigate the external factors that could influence the intestinal microbial composition such as antibiotics, or to test novel potential probiotics, before carrying out expensive *in vivo* trials. The data presented suggest that the potential probiotic strain *B. breve* B632 was able to survive in a complex microbial environment and restrained *Enterobacteriaceae* population. These results support the possibility to move to another level of study, i.e. the administration of *B. breve* B632 to a cohort of colicky newborns, in order to observe the *in vivo* behavior of this strain and to validate its effect in colic treatment.

4. Study of the bacteria involved in the intestinal proteolytic metabolism

4.1 Background

In the second part of this thesis project the proteolytic intestinal population in healthy adults has been characterized.

New generation high-throughput sequencing techniques were applied to obtain a comprehensive picture of the intestinal bacteria involved in proteolytic metabolism. In fact, for the majority of proteolytic bacteria within the gut microbiota, most information is available from culture-dependent studies. The experiments carried out on single cultivable species identified *Clostridiales*, *Bacteroidales*, and *Enterobacteriales* as the orders wherein many species fermenting proteins responsible of the phenolic compounds production can be found (Richardson et al., 2013). It is important to consider that each genus contains a variety of species which exert several different metabolic activities, that are characteristics of each single species and that depend on the environment in which these microorganisms live. In effect, the environment influence the characteristics of each organism and for this reason the behavior of a type strain could be different by its *in vivo* behavior. For this reason, the species involved in the intestinal proteolytic metabolism in a system delicately studied able to reproduce the intestinal environment have been investigated, and in a subsequent step, it will be possible to perform experiments with pure cultures, in order to study the physiology of these proteolytic species.

Three batch fermentations of intestinal microbiota were performed in a medium opportunely developed, containg only proteins and peptides as fermentable carbon source.

During the fermentations the following parameters have been monitored:

- total bacteria concentration,
- toxic metabolites: ammonium, p-cresol and indole,
- fermentation products: organic acids profile of volatile compounds,
- composition of the bacterial community by Illumina 16S sequencing

The last part has been conducted in collaboration with the Professor Andrès Moya, director of the FISABIO center of Valencia.

4.2 Materials and Methods

4.2.1 Fermentative process

A system based on batch fecal fermentations able to implement the growth of the bacteria responsible of the putrefactive proteolytic metabolism was developed in this phase of the study. During the experiments the cellular growth and the ammonium release were monitored. Moreover, the p-cresol, indole and the organic acids concentrations were evaluated. Finally, the composition of the volatile compounds profile was studied. The microbiota selected during the first 6 hrs of fermentation has been characterized through the sequencing of the rDNA 16S amplicons through Illumina technology.

3 batch fecal fermentations have been carried using different fecal samples and the bioreactors were inoculated at 1% w/v (concentration of the inoculum at time 0) .

4g of fresh fecal sample of the 8 healthy subjects were previously diluted 15 folds in Proteo Fermenter medium (PFM) through stomacher (Thermo Scientific) using an appropriate bag with a membrane able of containing the fecal debris. This medium properly formulated as reported in section 2.2.2, but the carbohydrates have been substituted with beef extract, sodium caseinate and peptones. These substrates were added as only source of energy using the following concentrations:

- Sodium caseynate 1.5g /L,
- Beef extract 1.5g /L
- Peptonenes 1 g/L,

The fermentations were carried out for 24 h. The temperature was set to 37°C and the culture was continually homogenized under gentile agitation at 150 rpm through a magnetic stirrer controlled by the software on line Iris V5 Infors. This software allows to control several parameters as temperature, gas flux and foam level. The pH was measure by InPro 3030 (Mettler Roledo, Swiss) electrode and was maintaint at 6.8 and through the automatic titration with HCl 4M and NaOH 4M.

During the experiments different samples were collected at 0, 6, 12 and 24 h. 23 ml of cellular suspension were collected and 20 ml were centrifuged for 10 minutes at 5500 rpm at 4°C and pellets and supernatant were frozen both at -80°C. The remaining volume was used to perform other analysis.

4.2.2 Enumeration of total cells number: DAPI

The DAPI (4′,6-Diamidino-2-phenylindole) is a fluorescent day that binds the region riches in A T of the DNA. It was utilized to count the cells (CFU/ml) in the counting chamber (Fertility Semen Counter Chamber, Hawksley). In order to count the cells it was used the fluorescent microscope Mikon ECLIPSE 80i.

100 µL of cellular suspension have been added to 100 µL of DAPI solution (5mg/L) in a final volume of 1000 µL reached through PBS pH 7.4. Sample was incubated in the dark for 15 minutes. After that, 5 µL have been withdrawn and charged into the counting chamber.

When necessary, 6 h after the inoculum, the sample was diluted.

4.2.3 Ammonium

Ammonium in the supernatants of the microbiota cultures was analyzed through a colorimetric assay, based on the formation of the indophenol blue using as reagents ‘Phenol nitroprusside solution’ (P6994 – SIGMA) and ‘alkaline hypochlorite solution’ (A1727 – SIGMA).

The supernatants were obtained centrifuging the cultures at 5500 rpm, for 10 minutes at 4°C. Samples were diluted 200 and 250 folds. The quantitative determination was obtained through the realization of a calibration curve in a concentration ranging between 0 and 150 µM.

Samples and standard were treated following the protocol reported below:

- 400 µL of the samples or standard were withdrawn and transferred in 50cologica,
- 400 µL of the phenol nitroprusside solution were added and the mixture were mixed,
- 400 µL of the alkaline hypochlorite solution were added and the solution was homogenized,
- samples were incubated at room-temperature for 30 minutes,
- the absorbance was appreciate at 625 nm (table 2).

The calibration curve, correlating absorbance (y) with ammonium µM concentration (x) is the following: $y = 0.014 \cdot x$ $R^2 = 0.99$.

4.2.4 Indole and p-cresol

The indole and p-cresol concentration in the supernatant of the fecal withdrawals were determined by HPLC (1100 Sistem (AGILENT TECHNOLOGIES). The instrument was equipped with a Diode Array Detector for the monitoring of the different wavelengths and Kinetex column (C18, 5 µm, 250x46 mm; PHENOMENEX).

1ml of the supernatant was centrifuged at 10 000 rpm for 10 minutes. 800µl were transferred into a vial and 10 µl were injected into the auto-sampler. For the elution of this two analytes it was applied a flux of 0.8 $ml \cdot min^{-1}$ obtained by mixing H2O + 0,1% formic acid and MeOH + 0,1% formic acid.

The analytes elution was monitored measuring the absorbance at 275 nm. The quantification has been possible thanks to the realization of a calibration curve ranging between 0-300 µM for both the analytes (table 2).

	M	R^2
Indole	4.17	1
p-cresol	1.16	0.99

***Table 2**: Slope (m) and R^2 of the linear regression for indole and and p-cresol*

4.2.5 Total carbohydrates

In order to exclude a contribute of the carbohydrates normally present in the fecal samples that could influence the experiment, the determination of the total carbohydrates was obtained through anthrone colorimetric assay.

Reducing or non reducing sugars are mixed with anthrone and sulfuric acid. This mixture generates a blu-green solution and the absorbance is measured at 620nm.

In order to have the quantification of the total sugars, the supernatants of the all time points were diluted when appropriate and processed through this method. The concentration was measured through the construction of a calibration curve between 0 and 150 $mg \cdot L^{-1}$.

The protocol is reported below:

- 500 ml of the fecal supernatant, fecal slurry or of the standard have been withdrawn and transferred in pre-cooled screw cap tube,

- 1000 µl of reagent (2 g/L of antrhone in H_2SO_4 96%) were added to the sample and vortexed,
- samples were incubated for 10 minutes at 100°C and quickly cooled in ice,
- absorbance measured at 620 nm (table 2).

The calibration curve, correlating absorbance (y) with carbohydrates mg/l concentration (x) is the following: $y = 0.018 \cdot x$ $R^2 = 0.99$.

4.2.7 SPME (Solid Phase Microextraction) and GC-MS analysis

The volatile compounds present in the supernatant of the fecal samples have been determined through SPME using the fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (Sigma Aldrich). The GC used is Agilent Technologies 7820° GC System with mass spectrometry Agilent Technologies 5975 series MSD.

2 ml of the supernatant have been transferred into a vial of 10 ml and 10 µL of HCl 10 M were added. The fiber was exposed for 1h to 60°C to the vial head-space. After that, the fiber was exposed for 15 minutes to the injector of the GC. The analytes are released through thermic disrobement and they are analyzed through DB-624 column (Agilent Tecnologies). The database used by the mass spectrometry is Nist08-Wiley09.

4.2.8 Sequencing of the 16S rDNA and 54cological54ics analysis

Amplification and sequencing

The genomic DNA of the cultures of each time points was extracted for the study of the composition of the microbial population most probably selected because of the selective pressure imposed during the experiments.

The DNA was extracted using the kit QIAamp DNA Stool Mini Kit (QIAGEN) following the protocol for the isolation of the DNA by pathogens.

Amplification, sequencing and bioinformatics analyses were carried out in collaboration with Prof. Andrés Moya of the FISABIO center of Valencia.

The DNA concentration was evaluated through Fluorometer Qubit® 3.0 (Life Technologies, Carlsbad, CA, USA). For each sample the concentration has been normalized at 5 ng/µL.

Amplicons for the genomic library have been generated using ribosomal primers for the 16S RNA. They create an amplicon of 460 bp on the V3 an V4 variable region.

- 16S Amplicon PCR Forward Primer:
5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;
- 16S Amplicon PCR Reverse Primer:
5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC;

The PCR reaction has been carried out in 96 Eppendorf™ Mastercycler™. The thermocycle consisted of 95°C for 3 minutes, followed by 8 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds with a final extension step at 72°C for 5 minutes.

The PCR products were cleaned through "Clean-UP by AMPure XP".

The libraries are quantified using "Qubit® 3.0 Fluorometer Protocol". Each amplicon was normalized at 4 nM and sequenced using MiSeq v3 Reagent Kit (Illumina).

Bioinformatic analyses

Bacterial raw sequences reads were filtered by size and quality using MOTHUR v.1.25.0. (Schloss, et al 2009). Sequence reads were assigned to their sample using a specific barcode-sequence and finally were linked in a unique fold.

Bacterial Identification and Taxonomic Attribution

QIIME pipeline 1.8.0 (Caporaso et al., 2010) was used to characterize the bacterial communities. Similar sequences were clustered into OTUs using UCLUST v1.2.22q (Edgar 2010) with an identity threshold of 97%. One sequence for each cluster was chosen as representative for that cluster using the most_abundant method.

The taxonomic attribution was performed through RDP Classifier 2.2 (Lan et al., 2012) employing the GreenGenes database release gg_13_05_otus at 99% identity. In order to obtain the phylogenetic tree relating the OTUs, frequently required for many downstream analysis, the representative sequences of each cluster were aligned with pre-aligned sequences (from GreenGenes) using PyNAST algorithm (Caporaso et al., 2010). These aligned sequences were filtered using a lanemask (lanemask_in_1s_and_0s from GreenGenes). Trees from multiple sequence alignments were constructed using FasTree method (FastTree version 2.1.7 SSE3).

The OTUs table and a folder containing taxonomy summary files (at different taxonomic levels) were generated. To calculate the absolute quantities, the relative abundance for each I was multiplied for bacterial concentration, obtained by DAPI enumeration.

Proteolytic OTUs were assessed measuring the difference between 6 h and 0 h samples. For each I, the number of duplications was calculated as the logarithm to base 2 of the increment. We used a cutoff of 3 duplications to choose OTUs associated to proteolytic energy provision.

4.2.9 Statistical analysis

All the chemical analysis were per formed in triplicate. The means of the values obtained have been analyzed through the statistical test T-Student and have been considered statistical different for $P < 0.05$.

4.3 Results and discussion

4.3.1 Proteolytic metabolism and amino acids fermentation in microbiota fecal cultures

Cultures of human gut microbiota were carried out in order to identify the main bacterial species involved in intestinal proteolytic metabolism based on amino acids fermentation yielding toxic metabolites.

The fecal microbiota from 3 healthy adults was inoculated into PFM broth, a specifically developed medium where proteins and peptones were the sole carbon and energy sources. The cultures were anaerobically incubated at 37°C for 24 h, with the pH maintained at 6.8. To monitor the occurrence of aminoacids fermentation, the concentration of ammonium, indole and p-cresol was periodically analyzed during the incubation. Ammonium is a general product of both the oxidative and the reducing parts of Stickland fermentations of aminoacids, while indole and p-cresol specifically originate from the fermentation of tryptophan and tyrosine, respectively. At the same time-points, bacterial counts were determined and total DNA of the culture was extracted to perform metagenome analysis of microbiota composition.

In all the cultures, the first hours were characterized by the most intense fermentation of the aminoacids and growth, with the major release of ammonium and indole. These compounds started to accumulate immediately after the inoculum and became stationary within 12 h in all the bioreactors. After 12 h of fermentation, ammonium ranged from 11 to 14 mM, while indole ranged from 73 to 75 M. A different behavior was observed for p-cresol, that was produced at negligible level in the culture inoculated with the microbiota of B1 (always < 2 µM), while it started to accumulate immediately after the inoculum in processes B2 and B3, even though production occurred at lower rate than indole and ammonium, and it did not reach a stationary level before the end of the fermentation (Figure 12).

In all the processes, an increase in total bacterial counts was observed in the first hours, leading to the highest concentration value at 6 h (B3) or 12 h (B1 and B2). Then, net growth stopped (Figure 12).

In order to determine whether the bacterial growth took place at the expenses of the undigestible polysaccharides and fibers introduced in the culture with the inoculum of feces, total carbohydrates were measured in the fecal samples utilized as inoculum and were monitored during the cultivation.

Carbohydrates, coming from a not complete digestion of some foods, are normally present into the fecal samples and could have an effect to the bacterial growth, promoting growth of saccharolytic bacteria. The average value of the total carbohydrates into the fecal samples (B1, B2 and B3) was 15 ± 1.6 mg/g, the soluble fraction being 3 ± 1.8 mg/g. The values corresponded to 0.15 and 0.03 g/l in the culture, respectively. In general, these compounds were quickly consumed in the time span of 0-6 h.

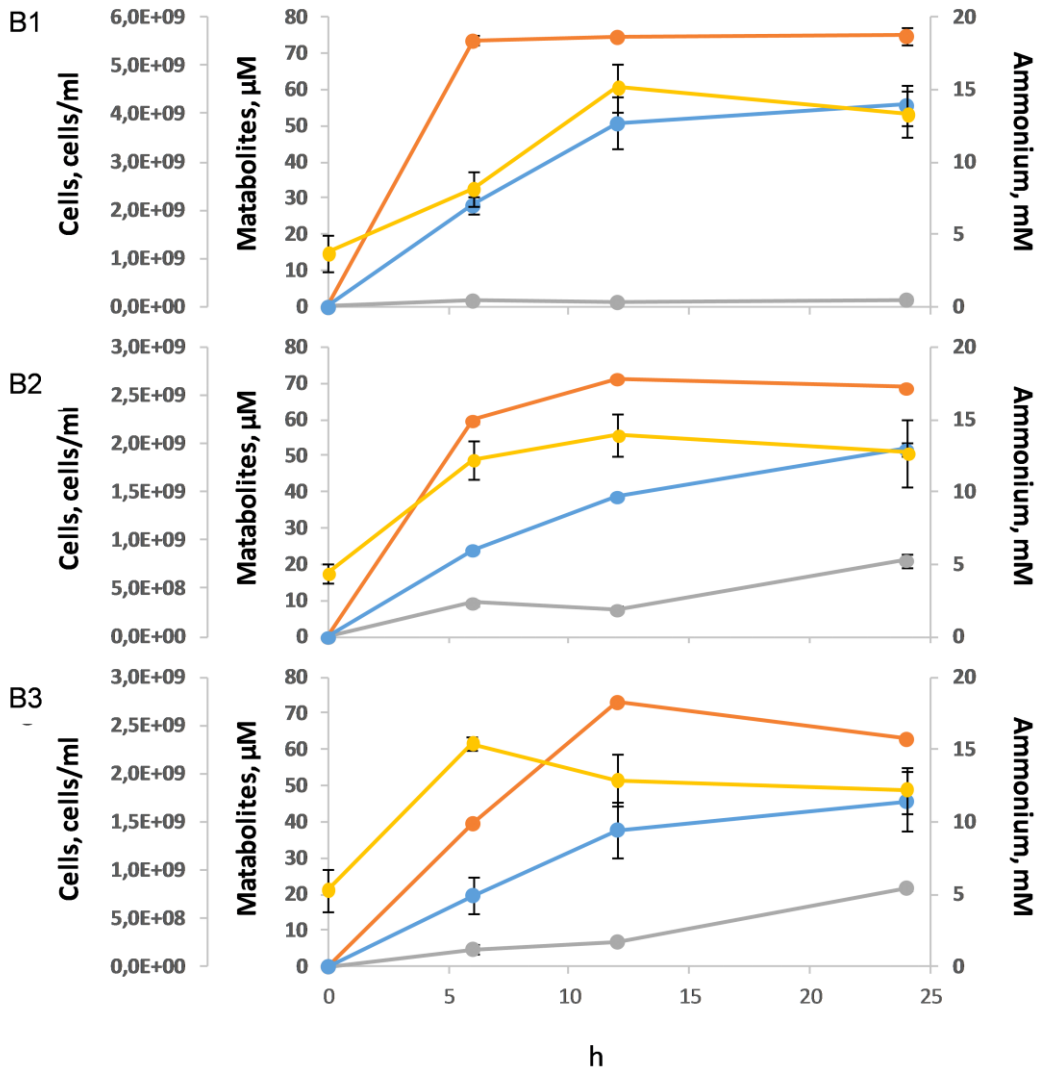


Figure 12: *Time-course of human intestinal microbiota cultures in a medium where proteins and peptones were the sole carbon and energy sources. Symbols:* ● *total cell counts;* ● *ammonium;* ● *indole;* ● *p-cresol. Values are means of 3 replications.*

The profile of the volatile compounds of each sample was determined at each time point by SPME GC-MS. CO_2 and 107 other volatile compounds were determined complexively, considering all the timepoints of all the fermentation runs (Table 3). Among them, 22 VOCs abundantly

accumulated in most of the fermentations during the first 6 h of incubation, when the metabolism of the culture was presumably mostly directed towards protein breakdown and aminoacid fermentation (Table 4), with a minimum overlapping of the re-utilization of fermentation products by other bacteria.

#	Compound	#	Compound	#	Compound
1	2-butanone	37	Benzenemethanol	73	4-octylbutan-4-olide
2	acetic acid ethyl ester	38	cyclohexanecarboxylic acid	74	Tetradecanal
3	l- cysteine sulfinic acid	39	octanoic acid	75	l-(-)-(z)-14-methyl-8-hexadecen-1-ol
4	n-methyltaurine	40	3,5,5- trimethyl- hexanoic acid	76	Hexadecanal
5	ethyl acetate	41	Decanal	77	pentadecanoic acid
6	acetic acid	42	isopropyl phenil ketone	78	Nonandecane
7	2- methylbutanal	43	benzoic acid	79	3-ethoxy-2- methyl-2-ciclopenten-1-one
8	1- butanol	44	2- ethyl- pieno	80	2,6-bis(1,1-dimethylethyl)-4-methyl-pieno
9	disulfide, dimethyl	45	3,5- dimethylbenzaldehyde	81	3,5-di-tert-butyl-4-hydroxybenzaldehyde
10	propanoic acid	46	1,4-bis(1,1-dimethylethyl)-benzene	82	13-octadecenal (z)
11	ethyl ester butanoic acid	47	Benzothiazole	83	2,5- dimethylbenzaldehyde
12	isopropoxycarbamic acid, ethyl ester	48	2,4- dimethylbenzaldehyde	84	1- docosanol
13	n-ethyl-1,3-dithioisoindoline	49	p-tert-buthyl-phenol	85	5- octyldihydro-2(3h)- furanone
14	propanoic acid, 2methyl	50	benzeneacetic acid	86	oxime methoxy-phenyl
15	2-methyl butanoic acid	51	nonanoic acid	87	dl-limonene
16	2-methyl propanoic acid	52	1,3-bis(1,1-dimethylethyl)-benzene	88	6-methyl-5-hepten-2-one
17	butanoic acid	53	2-ethoxy-4,5- dimethyloxazole	89	2 ethil texano
18	Furfurolo	54	[1,1'- bicyclopentyl]-2-one	90	benzene, 1,3-bis(1,1-dimethylethyl)
19	3-methyl butanoic acid	55	3-(1,1-dimethylethyl)-phenol	91	benzaldhehyde, 3,5-dimethyl
20	Heptanal	56	Benzeneacetaldehyde	92	5,9-undecadien-2-one, 6,10-dimethyl-, (e)
21	pentanoic acid	57	4-ethylbenzaldehyde	93	Cyclododecane
22	methoxy phenyl oxime	58	Indole	94	phenol, 2,4-bis(1,1-dimethylethyl)
23	1,2,3- trichloro-2- methylpropane	59	decanoic acid	95	1-tetradecene
24	Octanal	60	benzenepropanoic acid	96	1-pentadecanol
25	2- decanone	61	undecanoic acid	97	Cyclohexadecane
26	Benzaldehyde	62	2- methyl- indole	98	1-hexanol, 2-ethyl
27	4- methyl-2- heptanone	63	2,4-bis(1,1-dimethylethyl)-phenol	99	methyl 2-methyl-3-furyl disulfide
28	4- methylpentanoic acid	64	dodecanoic acid	100	dimethyl tetrasulphide
29	1-methyl-4-(1-methylethenyl)-cycloexene	65	hexadecanoic acid	101	2,5-cyclohexadiene-1,4dione,2,6bis
30	hexanoic acid	66	diethyl phtalate	102	phenol,2-4bis
31	1- (2-thiazolyl)- ethanone	67	tetradecanoic acid	103	benzaldehyde, 3,4-dimethyl
32	trisulfide, dimethyl	68	Dodecanal	104	propanal,3 (methylthio)
33	Pieno	69	1,13- tetrade diene	105	2-acethylthiazole
34	1- phenilethanone	70	11,13-dimethyl-12-tetradecen-1-ol acetate	106	diethyl phthalate
35	heptanoic acid	71	Octadecanal	107	2-tetradecene
36	4-methyl-phenol (p-cresol)	72	1- phenil-1- butanone		

***Table 3**: List of volatile compounds identified during the course of the fermentation runs in PFM medium. VOCs were analyzed by GC-MS analysis using SPME fiber. Compounds are numbered according to their elution order.*

Volatile compounds	
CO_2	3-methyl-butanoic acid
Acetate	Phenyl-acetic acid,
Propionate	phenyl-propionic acid
Butyrate	Benzoic acid
Valerate	p-cresol
Hexanoic acid (Caproic acid)	Indole
Octanoic acid (Caprylic acid)	3-methyl indole (skatole)
Decanoic acid	1-phenil-etanone
Dodecanoic acid	4-ethyl phenol
Isobutiyric acid	Decanal
4-methyl-pentanoic acid	3,5-dimethyl-benzaldehyde

***Table 4**: Main VOCs accumulated between 0 and 6 h.*

Acetate, propionate and butyrate are normal end products of the anaerobic fermentation of several complex molecules and they are generated during the proteolytic metabolism by the bacteria inhabiting the distal colon. They are also produced during the saccharolytic metabolism that takes place into the proximal colon (Scott et al., 2012). Valerate, isobutyric acid, 4-methyl-pentanoic acid and 3-methyl-butanoic acid are formed during the fermentation of valine, leucine, isoleucine and threonine (Nisman). Phenyl acetic acid is normally produced by the pathway related to the phenylalanine metabolism. The fermentation of the low-molecular-weight aromatic compounds and polyphenols coming from diet results in the production of benzoic acid (Manach et al., 2004). Pathways of the tyrosine and tryptophan metabolism, as extensively reported before, lead to the formation of p-cresol and indole and 3-methyl-indole (or skatole) (Windey et al., 2011). 1-phenyl-etanone and 3,5-dimethyl-benzaldehyde are normally produced by the fermentation of the aromatic amino acids (AAAs) (Windey et al., 2011). Hexanoic, octanoic, decanoic and dodecanoic acids are normally employed in various foods preparation (Fellows 2009). Finally, decanal is one of the main aldehydes in fermented foods (Fellows 2009).

4.3.2 Study of the microbial community in the time span 0-6 h

The composition of the microbiota cultures was analyzed by Illumina sequencing at 0 and 6 h of incubation in PFM medium. These timepoints were selected for comparison, based on the assumption that, during this time span, the cultures were forced to ferment proteins and peptones

as carbon source. Conversely, after 6h, following accumulation of organic acids and other fermentation products, other taxa able to use them as carbon source could be able to grow.

The raw reads obtained by Illumina sequencing were filtered for quality and chimeras, clusterized in OTUs at 97% of sequence similarity. To assign the taxonomic attribution to the OTUs, a sole sequence, the most represented, was chosen for each cluster. Taxonomy was assigned using a database clusterized at 99% of sequence similarity. Diverse OTUs assigned to the same taxon were pooled. At the end of the pipeline, a taxon table was created, reporting how many times a specific taxon appeared in each sample. The relative abundance was determined on the basis of the number of total reads, and a rough estimation of the number of bacteria belonging to this taxon per gram was get from DAPI counts. The bacterial abundance, expressed as the number of bacteria per ml of culture, was used to compare the concentrations at 0 and 6 h. The increment was calculated in terms of ratio (T6h/T0h), and the mean number of binary fissions that took place was determined as the base 2 logarithm to of the ratio. A threshold of 3 duplications was chosen to identify proteolytic OTUs associated to energy provision from amino acid fermentation.

As a whole, in the 6 samples coming from the two time-points of each of the three processes, 11692 OTUs were identified by the pipeline. This number was restricted to 268 taxa, summing up all the diverse OTUs ascribed to the same taxonomic unit. Proteolytic taxa were 30, 27, and 10 in B1, B2 and B3, respectively.

Overview of bacterial phyla in microbiota cultures

The figure below (Figure 12) reports the main phyla observed at 0 and 6 h in processes B1, B2, and B3. At 0 h, the PFM cultures inoculated with feces from the different subjects presented the same major bacterial groups. In all the subjects, the microbiota was dominated by *Bacteroidetes* and *Firmicutes*, together accounting for > 87% of total OTUs, followed by *Proteobacteria*.

Proteobacteria is the bacterial group that more than others increased its relative abundance after 6 h of incubation in PFM medium. In B1, they the 6.8% of the total reads at 0 h increased to the 20% after 6 h. In B2, this group represented the 2.1% at 0 h and 7.3% at 6 h. In B3 it was the 2.3 % and 7.1 % at 0 and 6 h, respectively. The relative bundance of *Firmicutes* remained almost constant even if in B2 their relative abundance slightly increased. Conversely, *Bacteroidetes* decreased their relative abundance after 6 h in all the cultures. At this taxonomic level the bacterial composition was studied considering all OTUs identified by the analysis (26 OTUs at phylum level).

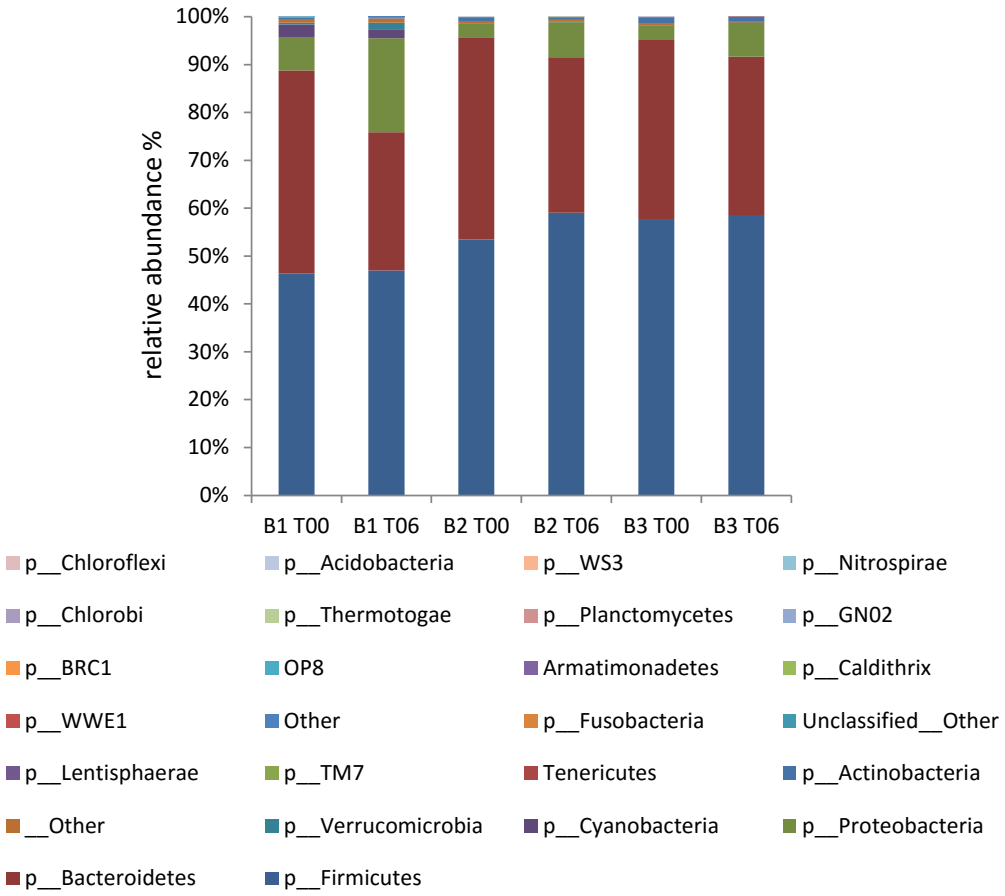


Figure 13: *relative abundance of bacterial phyla in cultures B1, B2, and B3 at 0 and 6 h.*

Taxa presenting at least 3 generations in 2 or 3 processes

Only the species ***Dorea fomicigenerans*** incremented in the three processes inoculated with different microbiota, performing 7, 5, and 4 generations in B1, B2, and B3, respectively. Other bacteria belonging to the genus ***Dorea*** increased only in B2 and B3, with 6 and 3 duplications, respectively. In B1 this increase was not appreciated likely because the initial concentration was much higher and, with few exceptions, for the most abundant species it was very hard to obtain several generations. *Dorea* spp. are members of the *Clostridium coccoides* group of organisms able to ferment glucose and several other carbohydrates, including polysaccharides and polyalcohols (Taras et al. 2002). Acetic, formic and lactic acids, ethanol, H_2 and CO_2 are the major products of glucose metabolism. They do not reduce nitrate to nitrite. *Dorea* comprises the two species *D. formicigenerans* and *D. longicatena*. *Dorea formicigenerans*, based on the study of Taras et al 2002, correspond to the previous spceies *Eubacterium formicigenerans* (Holdeman and Moore 1974). In general this species is normally found at large intestine and ileum levels as one

of the main species of the *Firmicutes* phylum (Hakansson and Molin 2011). The proteolytic metabolism has never been reported for *Dorea* species.

Bacteria belonging to the family ***Enterobacteriaceae*** grew in all the the cultures but they could not be taxonomically assigned at the level of genus or species. They performed 4, 6, and 5 in cultures B1, B2, and B3, respectively. *Enterobacteriaceae* are a large family of Gram-negative bacteria that includes many intestinal commensals, including some potentially harmful bacteria. Enterobacteriaceae are facultative anerobes which, in anaerobiosis, gain energy through mixed acid fermentation, where the products are a mixture of acids (e.g. lactate, acetate, succinate, and formate) as well as ethanol and equal amounts of H_2 and CO_2. Members of *Enterobacteriaceae* are well known as capable catabolizing aminoacids as carbon source (Sezonov et al. 2007).

Eggerthella lenta duplicated 3 and 4 times in fermentations B2 and B3, but it was never detected in B1. *Eggerthella lenta* is not able to oxidize or ferment carbohydrates (Yokoyama and Suzuky 2008). This species was previously classified as *Eubacterium lentum*. The genus *Eggerthella* belong to the family of *Coriobacteriaceae*, that is included into the phylum *Actinobacteria*, encompassing Gram-positive bacteria with high G+C content. *E. lenta* is one of the few species of the gut microbiota capable to transform the major soy isoflavone daidzein into S-equol, a powerful metabolite presenting high estrogenic and antioxidant activities. Equol has selective affinity for estrogen receptors, and in particular S-equol, the enantiomer produced by *E. lenta*, presents a high affinity for estrogen receptor β. Like *E. lenta*, also the other species involved in isoflavone transformations towards bioactive metabolites belong to the family of *Coriobacteriaceae*, suggesting a key role of this family in isoflavone metabolism into the mammalian intestine.

Ruminococcus callidus and ***Ruminococcus torques*** grew abundantly in B2 and B3 (6 and 4 generations in B2 and B3 respectively for R. *callidus*; 3 and 4 replications in B2 and B3 for R. *torques*). In B1, the former was very concentrated at the beginning of the process, an likely could not properly grow, and the latter grew remained below the threshold of three generations.

The genus ***Ruminococcus*** falls in the *Bacillus / Clostridium* subphylum of *Firmicutes*. The genus is not monophyletic, since it includes two clusters of species attributed to distinct families: ***R. callidus***, *R. flavefaciens*, *R. albus*, and *R. bromii* belong to *Ruminococcaceae* (i.e. the clostridial cluster IV), whereas ***R. torques***, ***R. gnavus***, *R. obeum*, *R. lactaris*, and *R. gauvreauii*, are misclassified since they belong to *Lachnospiraceae* (i.e. the clostridial rRNA cluster XIVa) (Collins et al., 1994; Rainey & Janssen, 1995). These latter species are therefore in need of reclassification and may form the nuclei of new genera (Liu et al. 2008).

True *Ruminococcus* spp. require ammonia and carbohydrates, but not amino acids as growth substrates. They play an important role in cellulose, hemicellulose, and pectin digestion and fermentation and has never been reported as able to ferment proteins (Rajilic-Stojanovic & Willem

de Vos, 2014). On the other hand, species such as *R. torques* and *R. gnavus* are among the most effective bacteria involved in breakdown and fermentation of mucins (i.e. O-linked N-acetylgalactosamine (GalNAc) glycoproteins that are major component of human mucus), with production of lactate, acetate and ethanol, with lower amounts of formate and succinate (Rajilic-Stojanovic & Willem de Vos, 2014). These latter species are among the most frequently isolated from the human intestinal microbiota, being widely distributed amongst individuals and being among the tens of species with high abundance in both adult and infant gut. Even though fermentation of the polysaccharidic chains of mucins by these species has been well characterized, information on the fermentation of proteins is not available.

Bacteria belonging to the family of ***Veillonellaceae*** increased in both B2 and B3 (4 and 3 generations respectively), but were not detected in B1. *Veillonellaceae* are the most numerous anaerobes in human saliva and among the most abundant organisms of oral and intestinal microbiota of animals and humans. Bacteria belonging to the genus *Veillonella* (The prokariotes pag. 1067) present a peculiar metabolism. They are not able to ferment carbohydrates, and obtain energy and carbon from organic acids such as lactate, pyruvate, malate, and fumarate by the key enzyme methyl malonyl-CoA decarboxylase, gaining energy from decarboxylation reactions (Dimroth 1985). The major end product of fermentative growth are propionic and acetic acids, carbon dioxide and hydrogen. Since lactate is one of the main fermentation products produced in the gut, *Vellionellaceae* exert a major role in cross-feeding relationship among colonic bacteria. Based on the current knowledge, it is hard to assess with definitively if they can ferment aminoacids, also if it has been demonstrated that *Vellionella* spp. can metabolize aspartate, threonine, arginine, and serine to acetate and propionate.

Sutterella increased in all bioreactors, performing 3 generations in B1 and B2, and 2 in B3. *Sutterellaceae* is a family within the order *Burkholderiales* in the lineage of phylum *Proteobacteria* (class *Betaproteobacteria*), including the genera *Sutterella* and *Parasutterella*. Members of the family are mainly found in the intestinal tract of mammals as members of the indigenous microbiota, and can be isolated from both the intestinal tract and from infections of gastrointestinal origin.

Others ***Burkolderiales*** increased in B1 and B2 (4 and 3 generations). The order *Burkolderiales*, comprises four families of Betaproteobacteria, some of which harbor species that have been implicated in human diseases such as respiratory infections (*Bordetella* spp.) and chronic granulomatous disease (*Burkholderia cepacia* complex spp.).

						B1		B2		B3	
phylum	class	order	family	genus	species	n	log_{10} cells g^{-1}	n	log_{10} cells g^{-1}	n	log_{10} cells g-1
Actinobacteria	*Coriobacteria*	*Coriobacteriales*	*Coriobacteriaceae*	*Eggerthella*	*lenta*			3	4.2	4	4.5
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	-			6	4.2	3	4.5
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	*formicigenerans*	7	5.1	5	5.9	4	6.5
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*callidus*			6	5.1	5	5.7
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*torques*			3	5.9	4	4.5
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	-	-			4	4.2	3	5
Proteobacteria	*Betaproteobacteria*	-	-	-	-	3	5.4	3	5		
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	-	-	-	4	4.9	4	4.2		
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Sutterella*	-	3	7.4	3	7.2		
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	-	-	4	7.1	6	5.4	5	5.6

Table 5: *Main bacterial taxa that increased in cultures B1, B2, and B3 during the period 0-6 h. Only taxa performing at least 3 generations in 2 or 3 fermentation runs are reported. For each OTUs, the initial concentration and the number of generations observed in the first 6 h of each batch are reported. Symbols: s, species, g, genus; f, family, o, order. N is the duplication number in the time span 0-6 h. For each OTUs is also reported the concentration estimated at time 0 expressed as Log OTUs* g^{-1}.

Taxa increased in B1 fermentation run

In total, 30 OTUs made 3 or more generations during the first 6 hours of process B1. Most of them belonged to the phylum *Proteobacteria* (15 OTUs), followed by *Firmicutes* (8 OTUs), *Actinobacteria* (3 OTUs), *Verrucomicrobiae* (2 OTUs), *Fusobacteria* (1 OTUs), and *Bacteroidetes* (1 OTUs).

Among *Actinobacteria*, bacteria belonging to the genera *Mobiluncus*, *Bifidobacterium*, and *Adelcreutzia* performed at least 3 generations in PFM medium. ***Mobiluncus*** belongs to the family of *Actinomycetaceae*, and is a recurrent colonizer of vagina, occurring in a large number of bacterial vaginosis (Bahar et al. 2005). ***Bifidobacteria*** are health promoting commensal saccharolytic bacteria. *Adlercreutzia* is a genus of asaccharolytic, obligately anaerobic *Coriobacteiaceae*, closely related to *Eggerthella*, normal inhabitant of the gut microbiota. Bacteria capable of converting soy isoflavones in to the potent phytosxtrogen belong to this genus (Maruo et al. 2008).

The phylum ***Proteobacteria*** encompassed diverse OTUs which increased in B1 cultures, performing at least 3 duplications. They belonged to the classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsiloproteobacteria*.

The species ***Acinetobacter lwoffii*** performed very well in the protein-based medium, and replicated 11 times. *A. lwoffii* belong to *Gammaproteobacteria*, within the family *Moraxcellaceae*. It presents variable virulence and can bear an impressive number of antibiotic- and toxin-resistance genes. Being ubiquitous in nature, *A. lwoffii* is part of the normal microbiota of oropharynx, human skin, and the perineum in approximately 20–25% of healthy individuals Other ***Gammaproteobacteria***, among which *Morexellaceae* and *Enterobacteriaceae* proliferated in B1, albeit it was not possible to assign them a taxonomy at genus or species level. Within ***Alphaproteobacteria***, the species ***Sphingomonas asaccharolytica***, performed 6 generations in PFM medium. ***S. asaccharolytica*** was described in 1990 by Yabuuchi et al. as Gram negative, strictly aerobic bacteria, mainly isolated from apple tree roots. Its genus contains 20 species that are metabolically versatile and are able to utilize a wide range of substrates and some of them could play an important role in human disease. However scarce information is available on the metabolism of *S. asaccharolytica*. Furthermore other ***Sphingomonadales*** performed 3 generations. In addition to the above mentioned genus *Sutterella*, other *Burkholderiales*, within ***Betaproteobacteria***, performed 4 duplications in the first 6 h in B1 cultures. Among ***Deltaproteobacteria*** were identified bacteria belonging to ***Desulfovibrionales***, which performed 4 generations. Within other ***Desulfovibrionales***, the genera ***Bilophila*** and ***Desulphovibrio*** could be identified, both perorming 3 generations. *Desulfovibrio* has a respiratory anaerobic metabolism that uses sulfate as final electron acceptor, with production of sulfide. *Desulfovibrio* normally uses lactate and pyruvate as electron donor, whereas the use of proteins as energy source has not been reported (Brayant et al., 1977). The genus *Bilophila* includes a sole species (*Bilophila wadsworthia*), strictly anaerobic, gram-negative sulphite reducing bacterium, unable to reduce sulphate. It is an important opportunistic identified in a multitude of anaerobic infections. It has a peculiar respiratory metabolism, where taurine is dissimilated with formation of sulphide, acetate, and ammonia. Taurine, deriving from taurine-conjugated bile acids, performs as source of sulphite, which is exploited as the terminal electron acceptor for the respiratory chain. This species is asaccharolytic, but it has never been described as proteolytic. Among ***Epsilonproteobacteria***, it was identified the species ***Campylobacter ureolyticus***, performing 4 generations. *C. ureolyticus* was previously classified as *Bacteroides urolyticus* (Vandamme et al. 1991). It has been isolated from patients with a range of diseases, including superficial ulcers, soft tissue infections, nongonococcal urethritis, perianal abscess, and gangrenous lesions, periodontal disease with inflammatory and destructive conditions of the tissues surrounding the teeth and it is knwn to produce putative virulence and colonization factors (Burgose-Portugal et al., 2012). Thus, it is considered as an emerging gastrointestinal pathogen, which promotes the secretion of proinflammatory cytokines and is likely involved in intestinal disease, such as Crohn disease and

ulceratice colitis. Metabolism of *C. urolyticus* has not been deeply characterized. It is anaerobic non-motile bacterium and as described by Cowan (1974); it is not able to grow on casein medium and the hydrolysis of this substrate is weak (Jackson & Goodman, 1978). It utilizes ammonia derived from urease activity, but it is not clear if it can ferment aminoacids.

The phylum *Firmicutes* included diverse OTUs which increased in the first 6 h in PFM medium. They belonged to the classes *Clostridia*, *Erysipelotrichi*, and *Fusobacteria*. Within ***Clostridia***, in addition to the above discussed species *D. formicigenerans*, were identified two families that grew abundantly on proteins were ***Peptococcaceae*** and ***Peptostreptococcaceae*** (6 and 8 duplications, respectively), both belonging to the order of *Clostridiales*. They are chemoorganotrophic with complex nutritional requirements. In general, they do not ferment carbohydrates and use peptone and aminoacids as major energy sources producing CO_2 and H_2 mostly from aminoacid fermentation. The major products of their metabolic pathway are acetic, butyric, isocaproic, lactic, propionic, isobutyric, and isovaleric acids. Another I for which it was possible to have a taxonomic assignment up to species level has been ***Oscillospira guillermondii***. It belongs to *Clostridia* class and *Firmicutes* phylum. The cultivation of this microorganism is not possible yet and it is the only species described in Bergey's Manual of Systematic Bacteriology. It is normally found in the rumen fluid of cattle and sheep (Mackie et al., 2003).

Within ***Erysipelotrichi***, bacteria of the family ***Erysipelothrichaceae*** performed 6 generations in B1. Few information is available concerning the function in the gut. Moreover, growth was observed also for ***Holdemania,*** a genus belonging to *Erysipelothrichaceae*. *Holdemania* have a fermentative metabolism, but it did not result proteolytic in prevous characterizations.

Within ***Fusobacteria***, growth was observed for bacteria of the genus ***Leptotrichia.*** *Leptotrichia* is part of the normal flora in the oral cavity, gut, and human female genitalia. This genus belong to the phylum *Fusobacteria* and to the family *Fusobacteriaceae*, and contains 6 diverse species representing a wide biodiversity. The phylum *Fusobacteria* includes Gram-negative, nonmotile, facultative aerobic to obligately anaerobic, fermentative, rod-shaped bacteria, which have generally fusiform morphology. *Fusobacteria* are commonly associated with the mucous membrane of humans and animals and they are significant constituents of the human oral cavity, playing an important role in periodontal disease. They ferment carbohydrates producing lactic acid. It has never been reported a proteolytic metabolism for *Leptotrichia* species.

Akkermansia muciniphila duplicated 4 times in 6 h. It belongs to the phylum ***Verrucomicrobia***, is strictly anaerobic, and utilizes mucins as carbon energy and nitrogen source (Darrien et al., 2004). Mucins are composed by high molecular mass glycoproteins and compose the mucus layer, that is an ecological niche for intestinal microbiota. This microorganism does not grow on carbohydrates and it is also able to release sulfate in a free form from mucin fermentation. It is not

expected to be saccharolytic (Darrien et al., 2004). Among *Verrucomicrobia*, also the family of ***Cerasicoccaceae*** got enriched during the first 6 h of this process.

One of the species identified by this study was ***Parabacteroides gordonii***. It performed 3 generations in the time span 0-6 h. This species belongs to ***Bacteroidetes*** phylum. ***Parabacteroides*** species are isolated mainly from human feces and clinical specimens. It is a strictly anaerobic microorganism, Gram-negative. Normally it grows on carbohydrates and the major end-products are acetic acid and small amounts of succinic acid (Sakamoto et al., 2009).

OTUs belonging to ***Molibuncus, Bifidobacterium, Adlerceruzia, Anaerofilum, Clostridia, Holdemania, Leptotrichia, Sphingomonadales, Bilophila, Moraxellaceae, Acinetobacteria,*** and ***Cerasicoccaceae*** increased 3 times only in this experiment.

Taxa increased in B2 fermentation run

In total, 27 OTUs made 3 or more generations during the first 6 hours of process B2. They belonged to the phyla *Actinobacteria* (1), *Bacteroidetes* (1), Caldithrix (1), Chloroflexi (3), *Firmicutes* (12), *Proteobacteria* (8), and *Verrucomicrobiae* (1).

Among Actinobacteria, only the above discussed *E. lenta* performed 3 generations. Within *Bacteroidetes*, only bacteria belonging to the family *Saprospiraceae* were found to grow in the first 6 h in PFM medium, performing 5 generations. *Saprospiraceae* consists of the genera *Aureispira, Haliscomenobacter, Lewinella* and *Saprospira*. The microorganisms belonging to this family are found in various habitats. They are anaerobics and Gram-negative and need of all the so called “essential amminoacids” (Bergey’s Manual of Systematic Bacteriology) to grow. The genera reported in literature were often isolated by marine environment and coastal zones (Saw et al., 2012) but currently, studies related to this group in the gastrointestinal tract of human are lacking. Bacteria included in the phylum *Chloroflexi* (Garrity & Holt 2001) increased by 3 generations. The phylum is recognized as a typical bacterial lineage that contains a number of environmental 16S rRNA gene sequences with a few cultured representatives (Rappé & Giovannoni 2003). It is divided phylogenetically into at least five major classes, including various photosynthetic or non-photosynthetic bacteria. In the present study, bacteria belonging to the family of *Caldilineaceae* grew of the protein based medium. Literature information is not available on the capability of *Caldilineaceae* to ferment proteins.

Among *Firmicutes*, bacteria belonging to the classes *Bacilli* and *Clostridia* grew in B2 cultures. Among *Bacilli*, growth was observed for the genus ***Turicibacter***. This genus is known to reside in the gastrointestinal tracts of animals and humans (Cuiv et al. 2011), suggesting that they may be

important members of the gut microbiota (e.g., references 4 and 7). However, only few strains were isolated and their physiology is poorly described .

In the class *Clostridia*, growth was observed for the families *Lachnospiraceae*, *Mogibacteriaceae*, *Ruminococcaceae*, and *Veillonellaceae*. Among *Lachnospiraraceae*, in addidion to the above discussed *Dorea*, the genus *Blautia* and the species *Coprococcus catus* were identified to perform 3 generations in the first 6 h. Based on polyphasic taxonomy, *Blautia* includes several microorganisms previously referred to as *Clostridium* or *Ruminococcus* (Liu et al., 2008). Bacteria belonging to Blautia spp. are coccoid or oval-shaped, obligate anaerobes with a saccharolytic fermentative metabolism yielding acetate, ethanol, hydrogen, lactate, and succinate as the end products of glucose fermentation. The species ***Coprococcus catus*** is an obligately anaerobic gram-positive bacterium. It is a normal inhabitans of the human intestinal microbiota and ferments carbohydrates through the acrylate pathway producing propionate. In *Ruminococcaceae*, ***Oscillospira*** spp., performed more than 3 generations, consisitently with the increase of *Oscillospira guillermondii* in process B1. Within the family *Veillonellaceae*, which includes intestinal anaerobes gining energy from shart chain fatty acids (Delwiche et al., 1985), the genus *Phascolarbacterium* increased during the first 6 h of process B2.

Among Proteobacteria, the species ***Hyphomicrobium zavarzinii*** grew only only in B2 process. It is a Hyphomicrobiceae, within the class of Alphaproteobacteria. This species is a major players in denitrification systems supplied with methanol as a carbon source. In general, *Hyphomicrobia* are facultative methylotrophs capable of using various reduced C-1 compounds, such as methanol, methylated amines, formate, or even halomethanes, methyl sulphates and methylated phosphates (Harder et al. 1978). *Proteobacteria* belonging to families ***Comamonadaceaea*** and ***Nitrosomonadeaceae*** yielded 3 generations only in B2 cultures, but were not classified at any deeper level.

Among Verrucomicrobia, an OUT of ***Pedospherales*** grew in the first 6 h, being found only in B2 cultures.

Taxa increased in B3 fermentation run

In B3 process 10 OTUs were found to yield at least 3 generations. Most of them belonged to *Firmicutes* (6), followed by *Proteobacteria* (2), and *Actinobacteria* (1).

All the *Firmicutes* that grew in PFM medium in the first 6 h belonged to the class *Clostridia*, in particular to families *Lachnospiraceae*, *Ruminococcaceae*, and *Veillonaceae*. The sole *Lachnospiraceae* were attributed to *Dorea*, including *D. formicigenerans*. The *Ruminococcaceae* were all attributed to the species *Ruminococcus*, particularly to the species *R. callidus*, and the

above discussed *R. gnavus*, and *R. torques*, each performing 4 or 5 generations. Within ***Veillonellaceae***, the species ***Veillonella dispar*** performed 3 generations. This species consists of small, obligately, anaerobic, Gram-negative cocci (Igarashi et al., 2009). It is isolated mainly from the intestinal tract of humans and the short-chain organic acids are its source of energy (Carlier, 2009). Other OTUs attributed to ***Veillonellaceae***, but not classified at any deeper taxonomy level, performed 3 generations.

Within *Proteobacteria* growth was observed for Enterobacteriaceae (5 generations) and for the species ***Helycobacter pylory*** (3 generations), the latter growing only in B3 process. H. pylory belongs to the class *Epsilonproteobacteria*. It is a gram-negative bacterium which colonizes the gastric mucosa of humans. It is a well-known pathogen involved in disease like gastritis and peptic ulcer. Moreover it is associated with certain types of gastric cancer. Doig et al., 1999 affirmed that the carbohydrate utilization is restricted to glucose and to sugars with shorter carbon backbones than C_6. It possesses numerous transporter systems for the uptake of amino acids, which must be available in the gastric environment. Therefore, other than carbohydrates, *H. pylori* prefers to use amino acids as important source of carbon.

Among Actinobacteria, the sole I that increased in B3 culture was attributed to the *E. lenta*.

phylum	class	order	family	genus	species	n	log_{10} cells g^{-1}
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Mobiluncus*	-	3	3.7
Actinobacteria	*Actinobacteria*	*Bifidobacteriales*	*Bifidobacteriaceae*	*Bifidobacterium*	-	3	5.4
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Adlercreutzia*	-	3	4.7
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Parabacteroides*	*gordonii*	3	4.0
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	*formicigenerans*	7	5.1
Firmicutes	*Clostridia*	*Clostridiales*	*Peptococcaceae*	-	-	6	3.7
Firmicutes	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	-	-	8	3.7
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Anaerofilum*	-	3	4.2
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillospira*	*guilliermondii*	3	3.7
Firmicutes	*Clostridia*	*SHA-98*	-	-	-	9	3.7
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*cc*	-	6	4.8
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Holdemania*	-	3	4.6
Fusobacteria	*Fusobacteriia*	*Fusobacteriales*	*Leptotrichiaceae*	*Leptotrichia*	-	3	3.7
Proteobacteria	-	-	-	-	-	4	5.4
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	-	-	-	3	3.7
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	*asaccharolytica*	6	3.7
Proteobacteria	*Betaproteobacteria*	-	-	-	-	3	5.4
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	-	-	-	4	4.9
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Sutterella*	-	3	7.4
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	-	-	-	4	3.7
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Bilophila*	-	3	5.9
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	-	3	6.7
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Campylobacter*	*ureolyticus*	4	3.7
Proteobacteria	*Gammaproteobacteria*	-	-	-	-	5	3.7
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	-	-	4	7.1
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	-	-	3	3.7
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	-	3	3.7
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*lwoffii*	11	3.7
Verrucomicrobia	*Opitutae*	*Cerasicoccales*	*Cerasicoccaceae*	-	-	3	6.6
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Akkermansia*	*muciniphila*	4	5.8

***Table 6**: Taxa that performed at least 3 generations in B1 process during the first 6 h. For each I, it is reported the deepest taxonomical level that could be determined, the number of generations (n), and the concentration at 0 h.*

phylum	class	order	family	genus	species	n	log_{10} cells g^{-1}
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Eggerthella*	*lenta*	3	4.2
Bacteroidetes	*Saprospirae*	*Saprospirales*	*Saprospiraceae*	-	-	5	4.2
Caldithrix	*KSB1*	*Ucn15732*	-	-	-	3	4.2
Chloroflexi	*Anaerolineae*	*Caldilineales*	*Caldilineaceae*	-	-	3	4.2
Chloroflexi	*Anaerolineae*	*GCA004*	-	-	-	3	4.2
Chloroflexi	*Anaerolineae*	*SHA-20*	-	-	-	3	4.2
Firmicutes	*Bacilli*	*Turicibacterales*	*Turicibacteraceae*	*Turicibacter*	-	3	4.2
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	-	-	3	7.4
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Blautia*		3	6.4
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*	*catus*	3	4.8
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	-	6	4.2
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	*formicigenerans*	5	5.9
Firmicutes	*Clostridia*	*Clostridiales*	*Mogibacteriaceae*	-	-	3	5.8
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Oscillospira*	-	3	7.3
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*callidus*	6	5.1
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*torques*	3	5.9
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	-	-	4	4.2
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Phascolarctobacterium*	-	3	7.2
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	*zavarzinii*	5	4.2
Proteobacteria	*Betaproteobacteria*	-	-	-	-	3	5.0
Proteobacteria	*Betaproteobacteria*	*ASSO-13*	-	-	-	3	4.2
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	-	-	-	4	4.2
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Sutterella*	-	3	7.2
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	-	-	3	4.2
Proteobacteria	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadaceae*	-	-	3	4.2
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	-	-	6	5.4
Verrucomicrobia	*Pedosphaerae*	*Pedosphaerales*	-	-	-	3	4.2

***Table 7**: Taxa that performed at least 3 generations in B2 process during the first 6 h. For each I, it is reported the deepest taxonomical level that could be determined, the number of generations (n), and the concentration at 0 h.*

phylum	class	order	family	genus	species	n	log_{10} cells g^{-1}
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Eggerthella*	*lenta*	4	4.5
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	-	3	4.5
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Dorea*	*formicigenerans*	4	6.5
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Ruminococcus*	*callidus*	5	5.7
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Ruminococcus*	*gnavus*	4	5.9
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Ruminococcus*	*torques*	4	4.5
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Ruminococcus*	-	3	5.0
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Veillonella*	*dispar*	3	4.5
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*	*pylori*	3	4.5
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	-	-	5	5.6

***Table 8**: Taxa that performed at least 3 generations in B3 process during the first 6 h. For each I, it is reported the deepest taxonomical level that could be determined, the number of generations (n), and the concentration at 0 h.*

4.4 Conclusions

The metagenomic 16S sequencing of samples from microbiota cultures growing in a medium containg proteins and peptones as sole carbon source lead to the identification of diverse taxa which abundantly grew, performing at least 3 generation in the first 6 h. In few cases, taxonomic attribution was deep enough to identify the species with a sufficient level of confidence, while in other OTUs were classified at a higher level (e.g. the genus, the family, or above). Some taxa grew in most of the fermentation processes, such as the species *Eggerthella. Lenta*, *Dorea formicigenerans*, *Ruminococcus callidus*, *R. gnavus*, the genera *Dorea* and *Sutterella*, and the families *Enterobacteriaceae* and *Veillonellaceae*. On the other hand, other taxa were specifically found in the diverse fermentation runs, inoculated with the microbiota from diverse subjects. It is the case of many genera and species that were identified in different experiments particularly within *Firmicutes* (e.g. *Turicibacter*, *Blautia*, *Oscillospira*, *Holdemania*, *R. torques*), *Proteobacteria* (e.g. *Sphingomonas asaccharolytica*, *Bilophila*, *Desulfovibrio*, *Campylobacter. Ureolyticus*, *Helycobactetr pylori*), and *Verrucomicrobia* (*Akkermansia muciniphila*). All the above taxa unequivocally are component of diverse microbiota, that are enriched when cultured in a protein/peptone-based medium. Nonetheless, not all the taxa could be unequivocally referred to as proteolytics or aminoacid fermenters. Based on available information about their metabolism, some taxa (for instance *Peptococcaceae* and *Peptostreptococcaceae*) are expected to ferment proteins and could have actually grown utilizing the proteins or peptides as carbon source. For other taxa (such as *Turicibacter* spp.), the metabolism was not investigated in sufficient detail to determine whether they could have effectively grown gaining energy from fermentation of proteins and aminoacids. On the other hand, literature excludes protein fermentation for some other taxa (such as *Holdemania* spp.) that performed diverse generations under these experimental conditions. These bacteria may have grown utilizing the fermentation products (e.g. the organic acids) produced by 'true' proteolytic members of the bacterial community, from which they depended in a cross-feeding relationship. Otherwise, they may have utilized some residual carbon source occurring in the fecal inoculum (e.g. the mucin and indigested fibers or polysaccharides), even though the initial amount of fermentable carbohydrate was negligible compared to proteins and peptides.

The present study obtained a picture of the bacterial diversity involved in protein breakdown within the colonic microbiota, utilizing new generation high-throughput Illumina sequencing. Yet, it is not conclusive in determining a functional/73cological role for the diverse bacterial taxa that were identified in enrichment cultures, in particular, with respect to the generation of harmful compounds from protein catabolism. To address this point, a thorough approach is necessary and

more work has to be done, including efforts in isolating the bacteria belonging to these taxa and characterizing their taxonomy and metabolism.

5. Composition of fecal samples from healthy subjects and patients affected by chronic kidney disease

5.1 Background

The chemical and microbiological composition of fecal samples of healthy subjects and patients affected by chronic kidney disease (CKD) was investigated. CKD entail a progressive loss in kidney function and is one of the most common chronic pathology in the developed countries. If not adequately treated, it can determine other pathologies as terminal kidney disease in which the patient needs a transplant. Patients affected by CKD suffer of a progressive loss of the organ functionality and are subjected to a gradual accumulation of toxic compounds in the organism. These toxins, called uremic retention molecules, are mailnly generated by gut microbiota and absorbed by the intestine. They can be final products of glycation reactions, and the major ones are p-cresol and indoles.

The metabolites not eliminated in patients affected by CKD are defined uremic toxins when (Vanholder et al., 2003):

1) they are chemically identified and can be quantified in the biological fluids;
2) the corporeal and plasmatic levels are higher in patients affected by CKD than in the healthy ones;
3) high concentrations are correlated with specific dysfunctions and symptoms, that decrease reducing the toxins concentration;
4) the biological activity is proved *in vivo* as well as *in vitro*.

The European Uremic Toxin (EUTox, http://www.uremic-toxins.org/) Work Group has classified these compounds in 3 major groups based on their chemical-physics properties influencing the dialysis:

1) Hydrosoluble compounds, with a molecular weight < 500 Daltons (Da); these compounds have not a highlighted toxic activity;
2) Compounds with an higher molecular weight (> 500Da), known as medium molecules;
3) Lyposoluble compounds and/or protein bound; normally they have a low molecular weight but some of them have medium molecules characteristics; they are toxic and is very difficult to remove them by the techniques normally employed (e.g. phenols and indoles).

Phenols and indols represent major uremic toxins, exerting relevant biological activities at several levels, with deleterious effects on human health. They are produced by bacterial proteolytic fermentation in the gut, and it is known that impaired intestinal barrier function in patients with

CKD permits translocation into the systemic circulation, contributing to the progression of CKD, cardiovascular disease, insulin resistance, and protein-energy wasting (Scott et al., 2013).

The metabolic activity occurring in the colon plays a pivotal role in production of these uremic toxins, since the last tract of the GIT contains both the precursors which can be fermented yielding indoles and p-cresol, and the bacteria that are involved in this transformation. While the field of gut microbiome research is evolving rapidly, less attention is paid to chemical composition of gut content. In this project the composition of fecal samples from healthy subjects and CKD patients was compared, and a further analysis of microbiota composition based on metagenomics approach is in progress.

For the two cohorts the following parameters have been determined:

a) fecal pH;
b) indole concentration;
c) p-cresol concentration;
d) ammonium concentration;
e) soluble carbohydrates concentration;
f) unsoluble carbohydrate concentration;
g) volatile compounds profile.

In particular, the composition of the fecal samples in cohorts of 10 healthy subjects and 8 patients affected by CKD was investigated.

Since indole and p-cresol production or degradation are expected to be associated to microbiota composition, the intra-individual and inter-individual variability of the indole and p-cresol formation / degradation kinetics in the healthy subjects was explored.

5.2 Materials and Methods

5.2.1 Samples collection

The experimental protocol (i.e. the utilization of feces for composition analysis and biotransformation assays) was approved by the institutional review board of the research ethics committee (Comitato Etico della Provincia di Bergamo, Italy).

The fecal samples belong to two different cohorts, the first composed by 10 healthy subjects and the second by 8 patients affected by CKD. All samples were collected in sterile containers and stored in anaerobiosis. For composition analysis, samples were frozen at -20°C until the use. For the indole and p-cresol transformations, samples were maintained at 4°C and utilized within 4 hours from collection.

5.2.2 Slurry preparation

In order to preserve the anaerobiosis in the slurries, dilution and homogenization steps were carried out insufflating CO_2. 10 g of feces were weighted in a bag and 10 folds diluted (w/v). The homogeneity of the suspension was reached by 2 minutes of mechanical break-up using the Stomacher device (Thermo). For pH measurement and chemical analysis 10% (w/v) fecal slurries were prepared using ddH_2O, while for transformation experiments the slurry was prepared in PBS pH 6.5 (Na_2HPO_4 1g/L, KCl 0.1g/L, KH_2PO_4 0.8g/L, NaCl 8g/L) containing tyrosine and tryptophan (each 200 µM) or indole and p-cresol (each 200 µM).

5.2.3 Chemical analysis

The pH of fecal samples and the amount of ammonium, carbohydrates, indole, p-cresol, and VOCs in fecal slurries were analyzed. When necessary for the specific analysis, supernatants of the slurries were obtained by centrifugation (18000 x *g* for 5 min at 4°C), followed by filtration at 0.22 µm.

The pH was analyzed on thawed fecal samples using an immersion electrode (CRISON). Ammonium was quantified in supernatants using phenol nitroprusside reaction (section 3.2.3). Total and soluble carbohydrates of the fecal slurries were determined by subjecting the fecal slurries and supernatants, respectively, to anthrone reaction (Trevelyan 1952). Indole and p-cresol

were quantified by HPLC-DAD (section 3.2.4). The profile of VOCs was determined through absorption onto SPME fiber and GC-MS analysis (section 3.3.7).

5.2.4 Kinetics of indole and p-cresol formation / degradation in resting-cells with the fecal slurry of the healthy volunteers

To determine the kinetic of indole and p-cresol formation or degradation, biotransformations with resting-cells of fecal populations were performed. For each fecal sample, three bioconversion reactions were monitored:

a) Control: fresh fecal sample diluted into the PBS pH 6.5;
b) Production of indole and p-cresol: fresh fecal sample diluted into the PBS pH 6.5 supplemented with the precursors tyrosine and tryptophan at the final concentration of 200 μM each;
c) Degradation of indole and p-cresol: fresh fecal sample diluted into the PBS pH 6.5 supplemented with indole and p-cresol at the final concentration of 200 μM each.

Samples were incubated anaerobically at 37°C. Samples were collected at 0, 4, 8, and 24 h and frozen at -20°C until analyzed for indole and p-cresol concentration.

5.2.5 Statistical analysis

All the chemical analysis were performed in triplicate. Data were analyzed through the Student's t-test and were considered statistical different for $p < 0.05$.

PCA was performed onto the profile of VOCs in healthy and CKD subjects. PCA is a technique for concentrating the information contained within a data set into a reduced number of statistically significant dimensions (Massart et al., 1997). This is achieved by creating new variables (the so-called Principal Components, PCs) that are linear combinations of the original ones (i.e., in our case, of the areas measured for the considered molecules) and which account for maximum possible variance in the data set. Each PC is constrained to be orthogonal (i.e., totally uncorrelated) with respect to all the previously extracted PCs, and as a consequence they have no overlap in information content. Each PC thus represents a different fundamental property of a system, were all the original variables that are partially or largely redundant in information content influence the

same PC in the same direction. This is evident in the loadings plot, which shows correlations between the PCs and the original variables. Moreover, it is possible to view how the objects of the data set (i.e., in our case, the different subjects) are distributed in the space of the PCs by means of the scores plot.

5.3 Results

Fecal samples from 10 healthy volunteers (labeled from A to L) and of 8 CKD patients (labeled from CKD1 to CKD8) were analyzed for pH, ammonium, VOCs, indole and p-cresol, in order to establish a comparison between the two cohorts. Moreover, to have an estimation of intra-individual variability, the healthy subject 'A' provided 3 fecal samples (referred to as A1, A2, and A3).

5.3.1 pH and ammonium

In the human gastrointestinal tract, protein fermentation takes place mainly in the distal colon where carbohydrates are depleted and proteolytic metabolism becomes dominant. Protein fermentation release ammonia, that determines the increase of the fecal pH from neutral or slightly acidic values (Nyangale et al. 2012).

	pH		pH
A1	7.31	**CKD1**	7.68
A2	6.18	**CKD2**	7.93
A3	6.98	**CKD3**	7.83
B	6.37	**CKD4**	6.24
C	6.75	**CKD5**	7.72
D	5.69	**CKD6**	6.37
E	7.05	**CKD7**	6.39
F	6.41	**CKD8**	6.65
G	6.44		
H	6.74		
I	6.86		
L	7.02		
mean	6.7		7.1
p-value	0.054		

Table 10: *pH of the fecal samples from healthy subjects (10 volunteers, 12 samples) and from CKD patients (8 subjects).*

In the cohort of healthy subjects, the pH of feces was 6.7 ± 0.5 (mean ± SD; Tab. 10), while in the CKD cohort it was 7.1 ± 0.8 (mean ± SD), without any significant difference ($p > 0.05$). The median values were 6.75 and 7.17 for healthy and CKD groups, respectively. The dispersion of these values was grater within the CKD cohort than into the healthy group (fgure 13). Albeit the absence of statystical significance, due to the low numerosity of the samples and to the wide dispersion of the data, the trend suggests a higher pH of fecal samples provided by CKD patients, likety due to a more proteolytic metabolism.

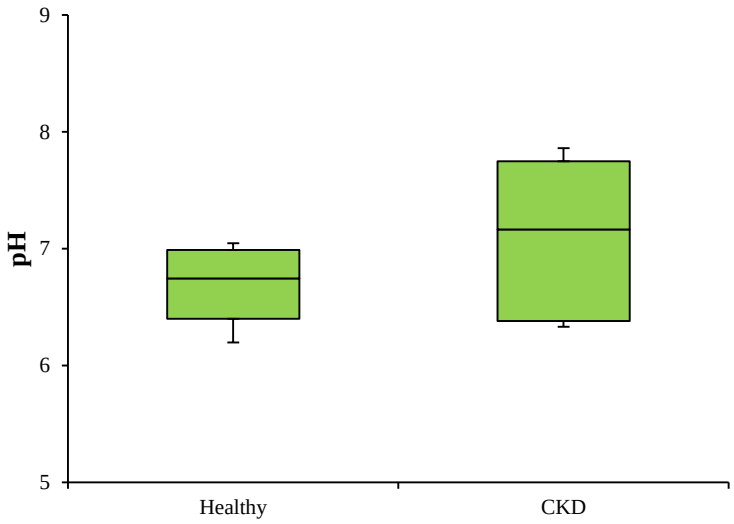


Figure 13: *Box-plots representation of the pH values of feces from healthy and CKD cohorts. The boxes represent the 25th percentile, the median, and the 75th percentile. Bars represent the 10th and 90th percentile.*

In the cohort of healthy subjects, ammonium concentration was 1352 ± 750 µg/g (mean ± SD; Tab. 11). It was significantly higher ($p < 0.05$) in CKD patients (3236 ± 2931 µg/g; mean ± SD). The median values were 1160.6 and 2207.4 µg/g for healthy subjects and patients, respectively (Figure 14). Likewise the pH, the CKD group presented a wider data distribution of ammonium values than the healthy group. The higher ammoninm concentration is consistent with the generally higher pH of feces from CKD patients.

	$NH4^+$		$NH4^+$
	µg/g		µg/g
A1	2019 ± 94	**CKD1**	1977 ± 172
A2	1980 ± 114	**CKD2**	1470 ± 267
A3	1255 ± 168	**CKD3**	1457 ± 251
B	859 ± 45	**CKD4**	4046 ± 474
C	869 ± 44	**CKD5**	3670 ± 634
D	3179 ± 221	**CKD6**	9958 ± 135
E	1465 ± 227	**CKD7**	2438 ± 722
F	827 ± 354	**CKD8**	874 ± 87
G	362 ± 240		
H	1409 ± 172		
I	933 ± 220		
L	1067 ± 161		
Mean	1352 ± 750		3236 ± 2931
p-value	0.023		

Table 11: *Ammonium concentration in the feces of healthy subjects (10 volunteers and one sampled 3 times) and CKD patients (8 subjects). Results are means ± SD (n=3). Statistical comparisons between the two groups have been measured through Student-t test.*

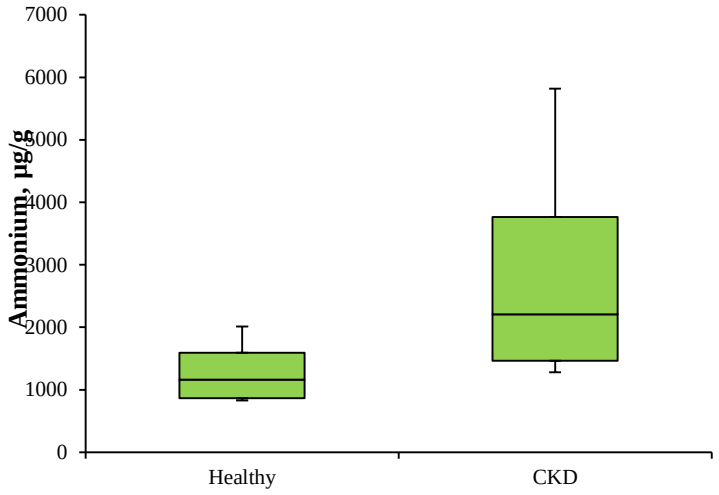


Figure 14: *Box-plots representation of the ammonium concentration values distribution within the 2 groups (healthy and CKD). The boxes represent the 25th percentile, median and 75th percentile. Bars represent the 10th and 90th percentile.*

5.3.2 Total and soluble carbohydrates

Total carbohydrates were 185 ± 143 mg/g in the feces of healthy subjects, lower ($p < 0.05$) than in the feces of CKD patients, (450 ± 392 mg/g; mean ± SD) (Tab. 12). The medians were 133.2 and 258.3 mg/g for healthy and patients cohorts, respectively.

	Total Carbohydrates mg/g_{DW}		Total Carbohydrates mg/g_{DW}
A1	97 ± 54	**CKD1**	277 ± 176
A2	31 ± 3	**CKD2**	157 ± 47
A3	51 ± 5	**CKD3**	150 ± 2
B	222 ± 2	**CKD4**	986 ± 58
C	238 ± 6	**CKD5**	1031 ± 160
D	101 ± 1	**CKD6**	688 ± 243
E	353 ± 9	**CKD7**	68 ± 6
F	528 ± 47	**CKD8**	239 ± 19
G	239 ± 20		
H	160 ± 11		
I	107 ± 42		
L	97 ± 4		
mean	185 ± 143		450 ± 392
p-value	0.023		

Table 12: *Concentration of total carbohydrates in the feces of healthy subjects (10 volunteers and one sampled 3 times) and CKD patients (8 subjects). Results are means ± SD (n = 3). Statistical comparisons between the two groups have been measured through Student-t test.*

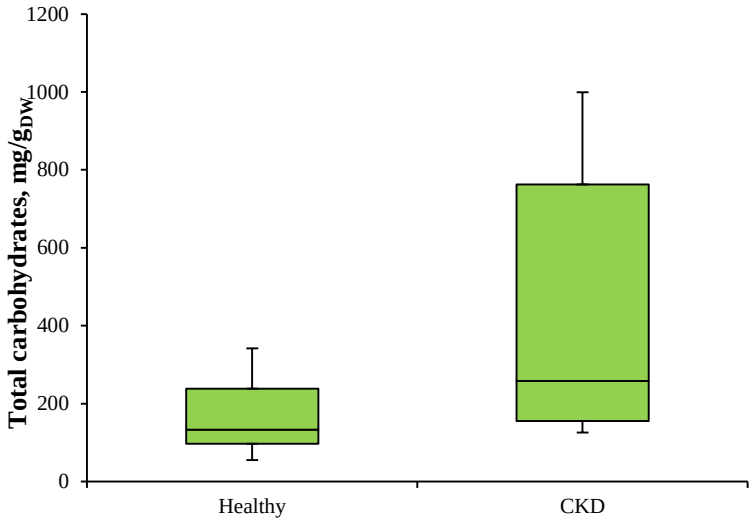


Figure 15: *Box-plots representation of the total carbohydrates concentration values distribution within the 2 groups (healthy and CKD). The boxes represent the 25th percentile, median and 75th percentile. Bars represent the 10th and 90th percentile.*

Concentration of soluble carbohydrates did not present significant differences ($p > 0.05$) between the two groups (Tab. 13). The concentration in the samples from the healthy subjects was 38 ± 47 mg/g (mean ± SD), while feces of CKD patients contained of 67 ± 49 mg/g of soluble carbohydrates. The median values were 25.3 and 55.0 for the healthy and patients cohorts, respectively (Figure 16).

	Soluble Carbohydrates mg/g_{DW}		Soluble Carbohydrates mg/g_{DW}
A1	13 ± 7	**CKD1**	36 ± 11
A2	5 ± 0	**CKD2**	18 ± 2
A3	7 ± 1	**CKD3**	13 ± 2
B	73 ± 7	**CKD4**	74 ± 18
C	21 ± 2	**CKD5**	143 ± 44
D	17 ± 0	**CKD6**	106 ± 19
E	30 ± 2	**CKD7**	33 ± 3
F	178 ± 43	**CKD8**	112 ± 11
G	29 ± 5		
H	39 ± 3		
I	16 ± 16		
L	31 ± 15		
mean	38 ± 47		67 ± 49
p-value	0.105		

Table 13: *Concentration of soluble carbohydrates in the feces of healthy subjects (10 volunteers and one sampled 3 times) and CKD patients (8 subjects). Results are means ± SD (n=3). Statistical comparisons between the two groups have been measured through Student-t test.*

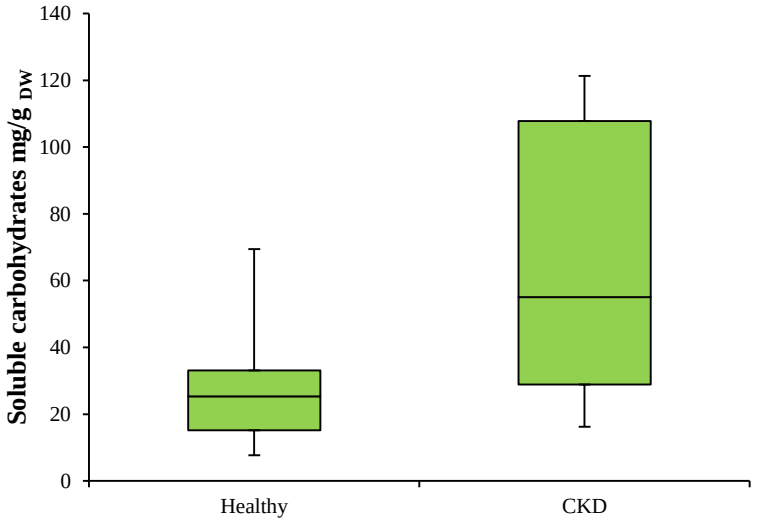


Figure 16: *Box-plots representation of the soluble carbohydrates concentration values distribution in the 2 groups (healthy and CKD). The boxes represent the 25th percentile, median and 75th percentile. Bars represent the 10th and 90th percentile.*

5.3.3 Indole and *p*-cresol

Concentration of indole and *p*-cresol was determined in the feces of healthy volunteers and CKD patients. In the feces of healthy subjects, indole was 70 ± 45 µg/g (mean ± SD), while in CKD samples it was 547 ± 1063 µg/g (Tab. 14). The dispersion of the latter cohort was particularly wide, mostly due to subjects CKD6 and CKD7 presenting remarkably high values of indole (783 and 3100 µg/g) and to subject CKD5, where indole was below the limit of detection. As a whole, significant differences of indole concentration were not observed between the two cohorts ($p > 0.05$).

	Indole µg/g		**Indole** µg/g
A1	52 ± 1	**CKD1**	69 ± 12
A2	81 ± 1	**CKD2**	210 ± 24
A3	36 ± 0	**CKD3**	24 ± 7
B	79 ± 10	**CKD4**	55 ± 15
C	110 ± 3	**CKD5**	0 ± 0
D	47 ± 3	**CKD6**	783 ± 20
E	187 ± 12	**CKD7**	3100 ± 140
F	23 ± 1	**CKD8**	133 ± 42
G	42 ± 0		
H	103 ± 0		
I	52 ± 0		
L	29 ± 0		
mean	70 ± 46		547 ± 1063
p-value	0.067		

***Table 14**: Concentration of indole in the feces of healthy subjects (10 volunteers and one sampled 3 times) and CKD patients (8 subjects). Results are means ± SD (n=3). Statistical comparisons between the two groups have been measured through Student-t test.*

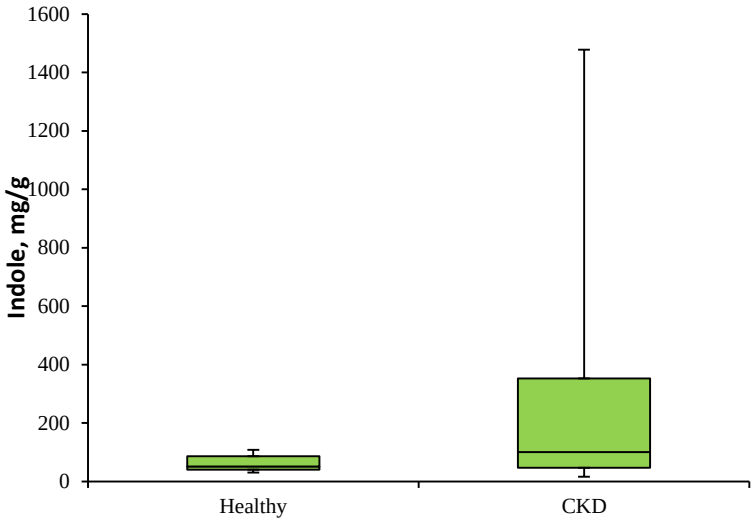


***Figure 17**: Box-plots representation of the indole concentration values distribution within the 2 groups (healthy and CKD). The boxes represent the 25th percentile, median and 75th percentile. Bars represent the 10th and 90th percentile.*

p-cresol was 332 ± 206 µg/g (media ± DS) in the feces of the healthy subjects and 767 ± 918 mg/g into the CKD samples (Tab.15). Median values were of 317.4 and 508.8, for healthy and CKD cohorts, respectively. Differences, als for this metabolite, were not significant ($p > 0.05$), likely because of the wide distribution of values within the CKD group.

	p-cresol ppm		p-cresol ppm
A1	755 ± 8	**CKD1**	847 ± 28
A2	368 ± 2	**CKD2**	98 ± 17
A3	304 ± 4	**CKD3**	613 ± 32
B	569 ± 12	**CKD4**	681 ± 24
C	166 ± 3	**CKD5**	371 ± 43
D	277 ± 2	**CKD6**	2950 ± 180
E	499 ± 20	**CKD7**	404 ± 22
F	71 ± 6	**CKD8**	173 ± 34
G	11 ± 0		
H	318 ± 2		
I	317 ± 0		
L	329 ± 0		
mean	332 ± 206		767 ± 918
p-value	0.063		

Table 15: *Concentration of p-cresol in the feces of healthy subjects (10 volunteers and one sampled 3 times) and CKD patients (8 subjects). Results are means ± SD (n=3). Statistical comparisons between the two groups have been measured through Student-t test.*

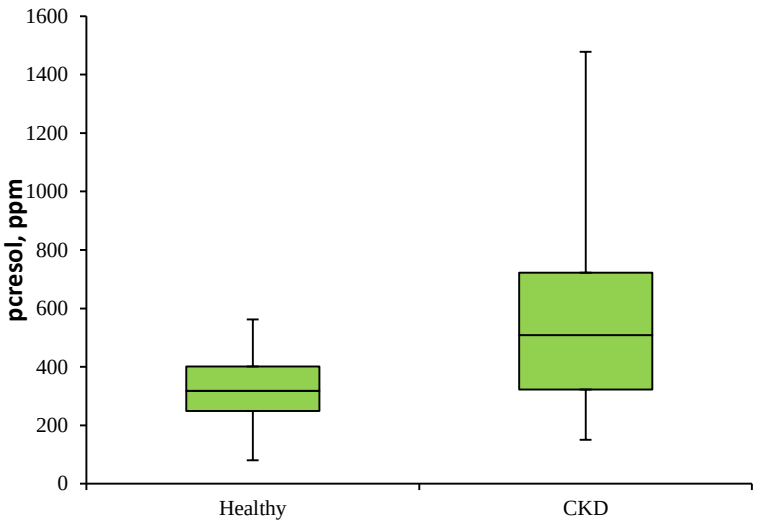


Figure 18: *Box-plots representation of the p-cresol concentration values distribution within the 2 groups (healthy and CKD). The boxes represent the 25th percentile, median and 75th percentile. Bars represent the 10th and 90th percentile.*

5.3.4 Formation and degradation of indole and p-cresol by the resting-cells of gut microbiota from healthy volunteers

Bioconversion experiments were carried out with resting-cells of intestinal microbiota from the healthy subjects, in order to determine the kinetics of formation and/or degradation of indole and p-cresol under different conditions. In particular, the bacterial cells were incubated without any addition (control), in presence of indole and p-cresol (IP), and in presence of their precursurs, tryptophan and tyrosine, respectively (TT) (table 17).

With the exception of sample G, all the controls yielded p-cresol, presumably utilizing some residual precursor occurring in feces. The precursor tyrosine in TT reaction mixtures determined a significant increase in the rate of p-cresol production in all the samples ($P < 0.05$), with exception of G. Production rates in presence of supplementary tyrosine ranged from to 0.1 to 2.1 $\mu mol/g_{DW}/h$, indicating a great inter-indiviudal variability in metabolic capabilities of diverse microbiota. This observation indicates that the precursor is limiting in control mixtures or that some tyrosine-dependent induction or activation mechanism is involved in p-cresol production. In IP reaction mixtures, the supplementation of p-cresol did not affect the rate of p-cresol production, compared to the control ($P > 0.05$). Therefore p-cresol did not exert any negative feedback on its generation and did not induce any mechanism leading to its transformation and/or removal. Therefore, p-cresol seems a recalcitrant mocecule which is produced, but not degreaded by the gut microbiota under these experimental conditions, always presenting a positive balance.

A slight basal production of indole occurred in most of the samples, under control conditions. Likewise p-cresol, the presence of the precursor tryptophan in TT mixtures yielded a significant increase in the production rate ($P < 0.05$), similarly indicating that the precursor is limiting in control mixtures or that it is necessary to induce or activate the biotransformation. Likewise p-cresol, the rates of indole production in presence of supplementary triptophane were quite different among subjects, ranging from to 0.4 to 4.4 $\mu mol/g_{DW}/h$, confirming a high inter-indiviudal variability in metabolic capabilities of diverse microbiota. Unlike p-cresol, the presence of indole in IP mixtures lead to negative values for its variation rate, indicating that degradation is more relevant than production under these conditions. These results suggest that the microbiota of the healthy subjects is able to both degrade or produce indole, and that the overall balance depends by the availability of indole and/or triptophane.

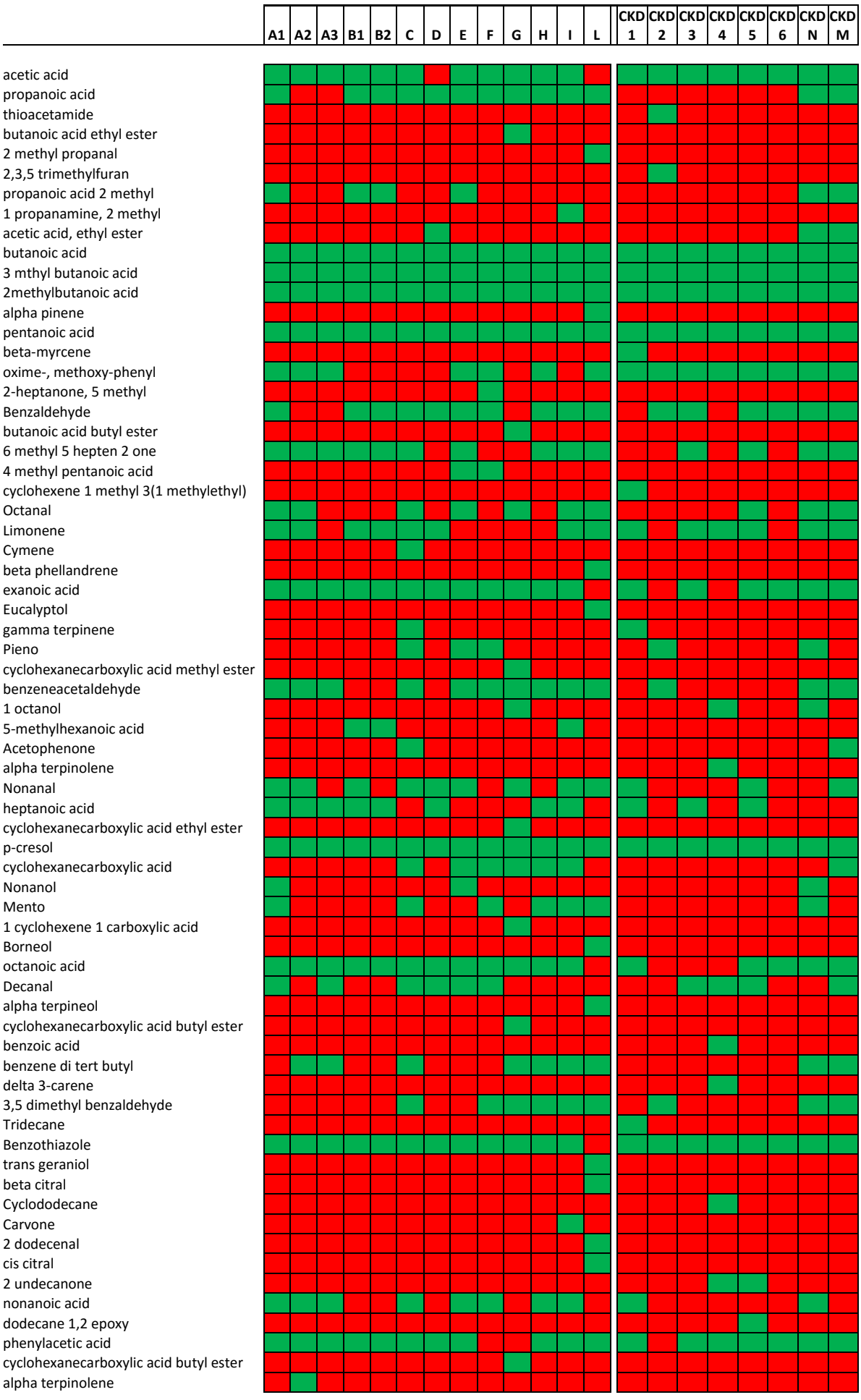
Any linear relationship between the above reported indole and p-cresol concentrations and the respective production rates under control and IP conditions was not observed (R^2 always < 0.37).

	p-cresol			Indole		
Samples	**control**	**IP**	**TT**	**control**	**IP**	**TT**
	µmol/g_{DW}/h			**µmol/g_{DW}/h**		
A1	0.3	0.2	1.3	0.1	0.1	0.6
A2	0.3	0.2	0.7	0.0	-0.1	0.5
A3	0.1	0.2	0.5	0.0	-0.1	0.4
B	0.2	0.4	1.6	0.1	-0.3	3.7
C	0.2	0.2	2.1	0.1	0.0	2.6
D	0.4	0.3	0.9	0.1	-0.1	1.1
E	0.3	-0.1	1.6	0.1	-0.3	3.1
F	0.0	0.0	0.1	0.4	-0.1	4.4
G	0.0	0.1	0.0	0.1	0.1	0.9
H	0.4	0.2	1.7	0.0	-0.1	2.8
I	0.3	0.4	1.3	0.1	0.1	1.5
L	0.4	0.2	1.8	0.0	0.0	2.0
mean	0.3*	0.2*	1.1	0.1	-0.1	2.0
SD	0.1	0.1	0.7	0.1	0.1	1.3

Table 17: *Rate of variation of indole and p-cresol, catalyzed by resting-cells of gut microbiota without any addition (control), in presence of indole and p-cresol (IP), and in presence of tryptophan and tyrosine (TT). Results are means, n = 2, DS always < 0.005. Groups were compared with paired-samples Student's t-test and considered different for P < 0.05. Means are significantly different, unless they share the same symbol.*

5.3.5 Profile of volatile compounds

Approximately 150 compounds were observed in the headspace of acidified fecal slurries, in particular SCFAs, ketones, aldehydes, hydrocarbons (C_{10}-C_{16} saturated and unsaturated), indole, skatol, p- cresol, and terpenes (Table 16). The green box indicates the presence of a specific compound in a fecal sample, the red one identifies the absence of that compounds into the fecal sample.


A1
A2
A3
B1
B2
C
D
E
F
G
H
I
L
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5
CKD 6
CKD N
CKD M
acetic acid
propanoic acid
thioacetamide
butanoic acid ethyl ester
2 methyl propanal
2,3,5 trimethylfuran
propanoic acid 2 methyl
1 propanamine, 2 methyl
acetic acid, ethyl ester
butanoic acid
3 mthyl butanoic acid
2methylbutanoic acid
alpha pinene
pentanoic acid
beta-myrcene
oxime-, methoxy-phenyl
2-heptanone, 5 methyl
Benzaldehyde
butanoic acid butyl ester
6 methyl 5 hepten 2 one
4 methyl pentanoic acid
cyclohexene 1 methyl 3(1 methylethyl)
Octanal
Limonene
Cymene
beta phellandrene
exanoic acid
Eucalyptol
gamma terpinene
Pieno
cyclohexanecarboxylic acid methyl ester
benzeneacetaldehyde
1 octanol
5-methylhexanoic acid
Acetophenone
alpha terpinolene
Nonanal
heptanoic acid
cyclohexanecarboxylic acid ethyl ester
p-cresol
cyclohexanecarboxylic acid
Nonanol
Mento
1 cyclohexene 1 carboxylic acid
Borneol
octanoic acid
Decanal
alpha terpineol
cyclohexanecarboxylic acid butyl ester
benzoic acid
benzene di tert butyl
delta 3-carene
3,5 dimethyl benzaldehyde
Tridecane
Benzothiazole
trans geraniol
beta citral
Cyclododecane
Carvone
2 dodecenal
cis citral
2 undecanone
nonanoic acid
dodecane 1,2 epoxy
phenylacetic acid
cyclohexanecarboxylic acid butyl ester
alpha terpinolene

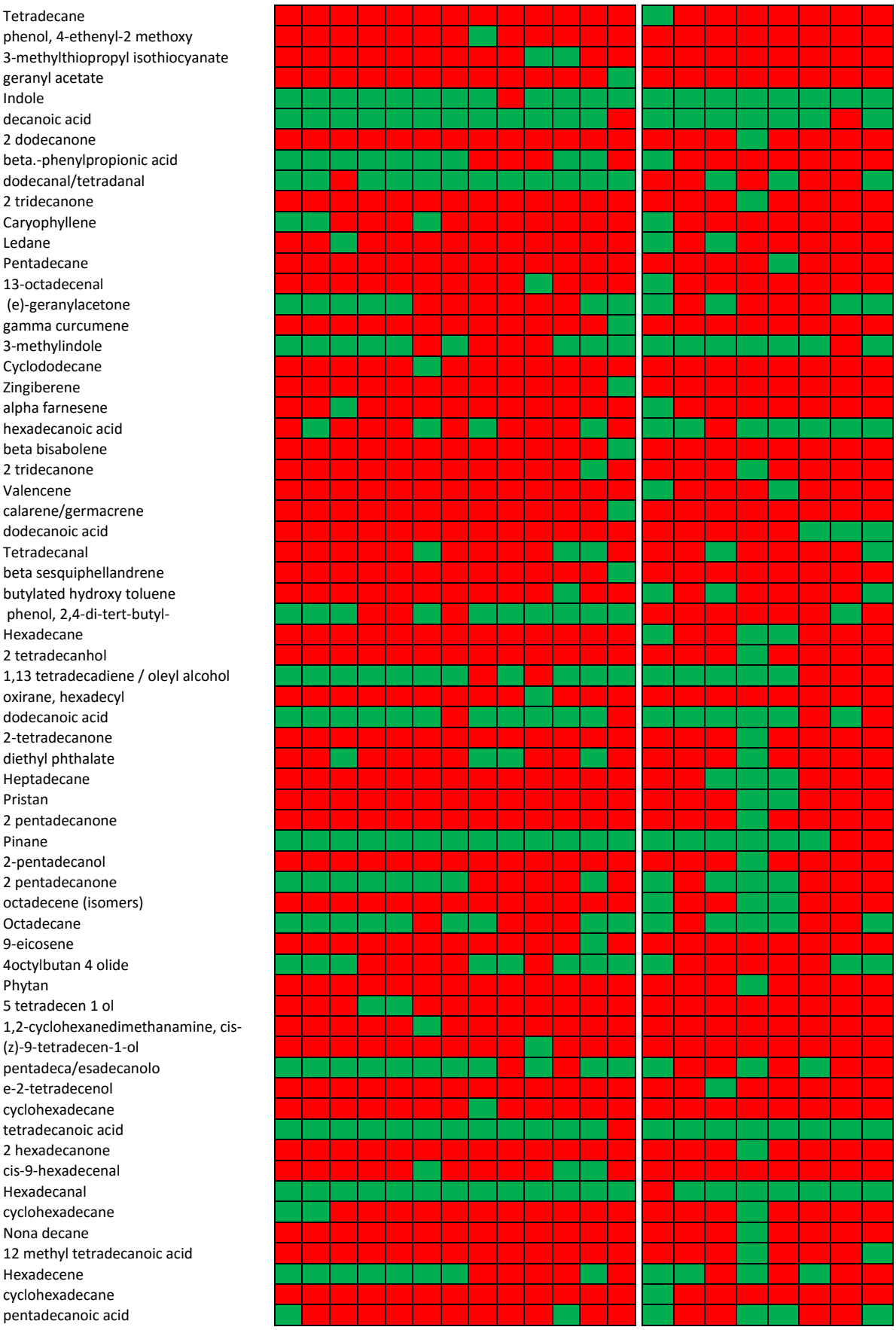


***Table 16**: compounds observed in the headspace of acidified fecal slurries. Metabolites have been identified SPME fiber analyzed through CG-MS. Compounds are ordered according to their elution.*

Data obtained from SPME GC-MS were subjected to Principal Components Analysis in order to highlight the differences between the two groups. The criterion of the average eigenvalue was used in order to choose the number of principal components. Observing the scores plot is possible to note that, in general, samples differ for PC1 that represent 20.64% of the data variability (PC2 explains the 14.93 %). Two main cluster could be identified even if some samples belonging to healthy groups (B, D, A1, A2, and A3) clusterize with samples of CKD patients cohort. The comparison of scores vs loadings plots is possible reveals that the group in the upper right part of the plots (samples I, H, and C) is characterized by higher amounts of oleyl alcohol, dodecanoic acid, octanoic acid, tetradecanal, hexanoic acid, heptanoic acid, diethyl phthalate, and phenol 2,4 di-tert-butyl. Samples grouped in the lower right (CKD7 and CKD8) present higher amounts of 3-methyl-butanoic acid, 2-methyl-butanoic acid, hexanoic acid, propanoic acid 2 methyl, benzaldehyde, and octanal benzene-di-tert-butyl. CKD6 has higher amounts of p-cresol, phenylacetic acid, oximemetoxy-phenyl and indole. The other variables are not able to explain any variability (loading very close to 0).

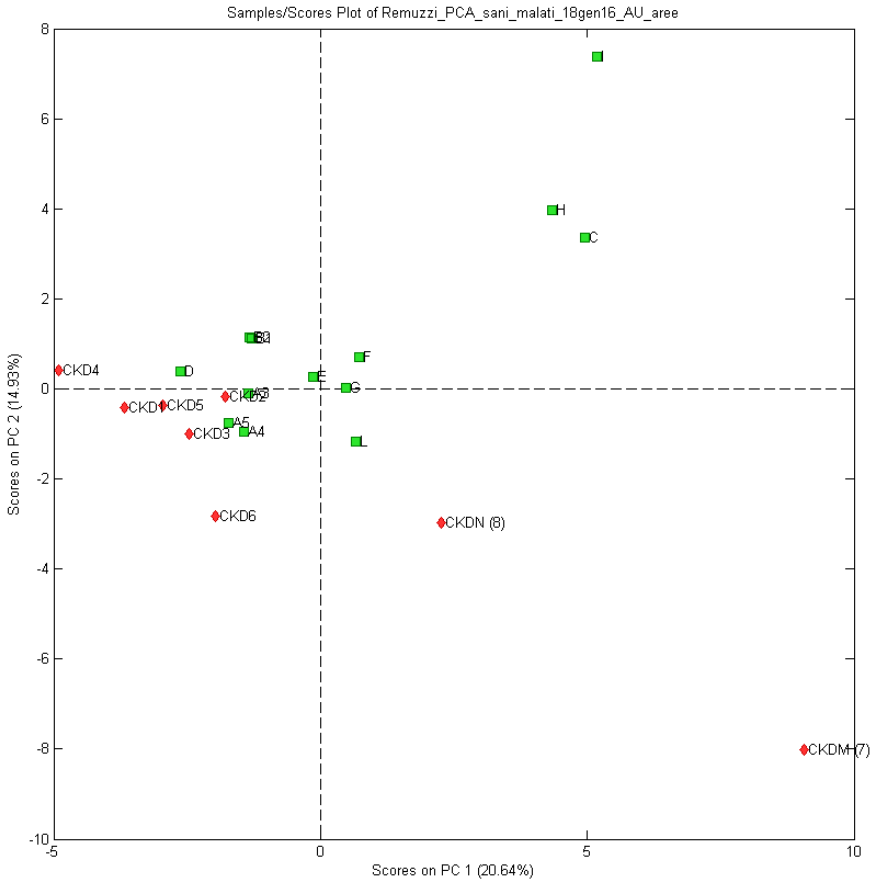


Figure 19: *scores plot of the volatile compounds profile made through the Prencipal Component Analysis (PCA)*

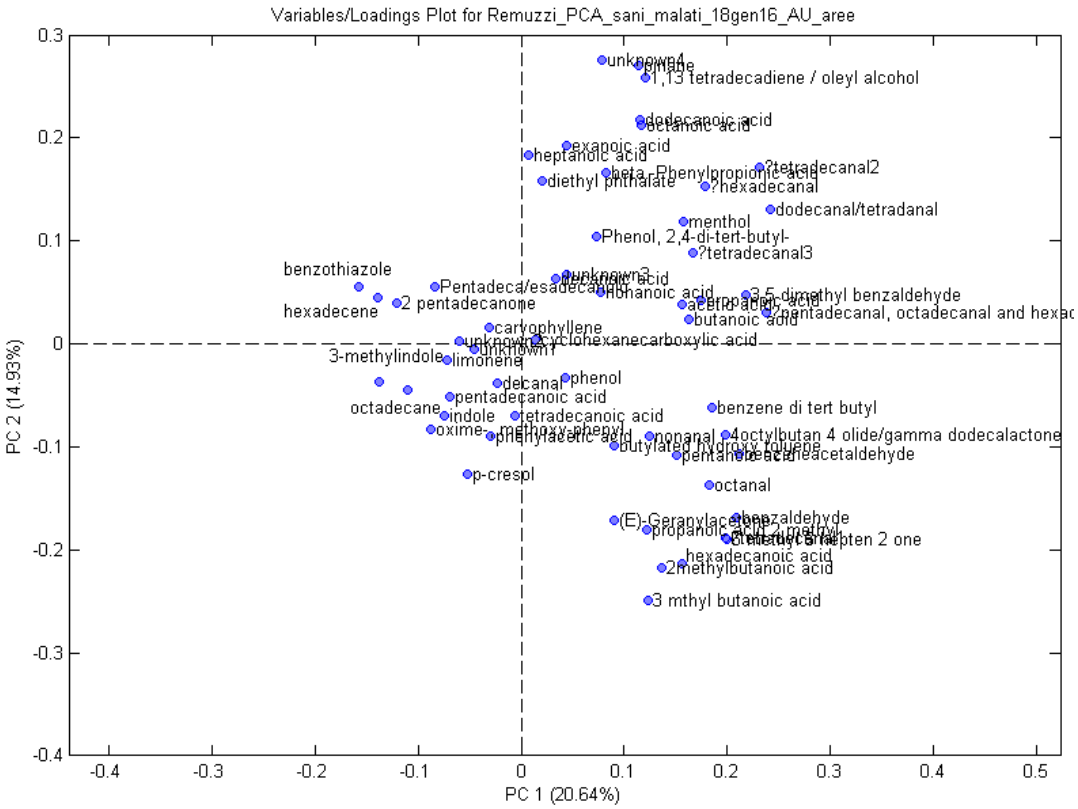


Figure 20: *loadings plot of the volatile compounds profile made through the Prencipal Component Analysis (PCA)*

5.4 Discussion

In the last decade, major attention has been focused on microbiota description. However, information of feces composition is not yet available. In particular, feces can be regarded as an intriguing matrix composed by gut microbiota, undigested materials, and products of metabolic activity of bacteria. Then, it is very important to shed light into chemical composition of fecal samples, also in order to associate these data to metagenomics investigations. In this project, we compared the composition of fecal samplesa from healthy subjects and from CKD patients, determining the pH and the concentration of ammonium, indole, p-cresole, soluble and insoluble carbohydrates. The metabolites indole and p-cresole were analyzed since they are an important marker of proteolytic metabolism. Colonic microbiota expresses proteases and peptidases that have a role in degradation of dietary and endogenous proteins that reach the colon undigested (Mcfarlane et al., 2009). The aromatic aminoacids phenylalanine, tyrosine and tryptophan, released through this process, are subsequently fermented with production of indolic and phenolic compounds, that tend to accumulate into the distal colon where saccharolytic metabolism decreases. Indole and *p*-cresol are by the epithelial colonic cells, transferred into the bloodstream, metabolized in the liver, then excreted in the urine where they represents a major portion of the phenolic compounds. When renal disorders occur, these metabolites accumulate and exploit a toxic effect on several tissues and organs, including kidney. High *p*-cresol concentrations into the serum compromise the endothelial function, and they are normally used as marker for cardiovascular diseases (Meijers et al.,2009).

In our study, between the groups of healthy subjects and CKD patients, no significant differences in pH were found, albeit a lower pH of the feces of the healthy subjects was detected. Consistently, ammonium amounts were significantly different between the two groups, higher in feces from CKD patients. This is probably related to a more active proteolytic metabolism into the distal colon, since ammonium is released in the first step of the amminoacid fermentation carried out by proteolytic bacteria. The higher concentration of carbohydrates of the fecal samples from CKD patients can derive from a less efficient saccharolytic population, that does not ferment and assimilate completely the sugars. Normally, carbohydrates support growth of saccharolytic bacterial groups, and contribute to the decrease the fecal pH. Then, it is possible that CKD disease affects microbiota, and promote proliferation of proteolytic groups (Evenepoel et al., 2009 and Varziri et al., 2013).

Consisteltly, ammonium and total carbohydrates concentrations were significantly different between the two groups, both being higher in CKD patients. This result confirms that a more efficient proteolytic metabolism takes place into the distal colon of CKD patients, with a likely less performing saccharolytic microbiota, that are not able to ferment a relevant part of the unsoluble carbohydrates.

Indole and *p*-cresol concentrations were not significantly different between the two groups, presumably because of the low numerosity and of the wide interindividual variability for both metabolites, within the two groups.

The volatile compounds present in feces have been analyzed through SPME GC-MS, and the data have been processed by PCA analysis. Samples from CKD patients and healthy subjects do not form two definite clusters. In fact, the samples B, D, A1, A2, and A3 clusterize with the CKD patients cohort. Some metabolites are produced mainly by the healthy volunteers (in the upper right part of the loadings plot). Conversely, the fecal samples of the patients CKD 4, CKD 6, CKD 7, and CKD 8 are characterized by higher amounts of benzene di-tert-butyl, benzothiazole, 3-methyl-butanoic acid, 2 methyl-butanoic acid, p-cresol, and 3-methyl-indole, metabolites generally produced by the fermentations of amminoacid.

The efficicncy of gut microbiota to produce or transform indole and *p*-cresol has been studied only with the intestinal microbial populations of healthy subjects, in order to determine the range if the interindividual variation. The outcome of these preliminary experiments indicates that *p*-cresol is poorly degraded while the indole transformation seems to be induced by its high concentrations. Both these metabolites accumulate quickly in the presence of their respective precursors tryptophan and tyrosine.

6. Conclusions

In this Ph.D. project, fermentation processes inoculated with human gut microbiota were utilized to study the efficacy of a novel probiotic strain and to enrich the gut microbiota on a protein-based medium. The gut population is a highly diverse community, composed mainly of bacteria. Metagenome survey, based on high throughput sequencing techniques, is the most powerful tool for obtaining a picture of a bacterial community where the majority of the members are considered unculturable. Besides, culturing gut microbiota offers several advantages for analysing the function and the metabolism of gut bacteria.

Bioreactor fermentation processes, associated to different culture-dependent and independent techniques for beacterial enumeration, made it possible to assess the inhibitory effect of ta novel *Bifidobacterium* probiotic strain against Enterobacteriaceae. The outcome of this study is very promising in the perspective to develop targeted probiotic strains able to alleviate the symptoms of gaseous colics in the newborns. In fact, literature is consistent in identifying coliforms and eneterobacteriacee as major responsible of this disease, and probiotics, albeit not specifically targeted, are already used with success to limit colics. The possibility to perform competition experiment *in vitro* allowed the collection of preliminary promising data, with the perspective to perform a trial *in vivo*.

A similar *in vitro* model was utilized to culture the microbiota of 3 adult healthy subjects in a medium containing only proteins and peptones as carbon source. This strategy forced the growth of bacteria gaining energy from protein breakdown, albeit several other taxa that are not able to ferment proteins performed some generations as well. Three fermentations runs, inoculated with the feces of different subjects, resulted in the enrichment of diverse taxa, with a limited core of common bacteria proliferating in the three process. However, most of the species, genera, and higher leve taxa that increased by at least three generations were different among the three samples, suggesting that each microbiota presented a peculiar proteolytic population, that took advantage in these growth conditions. Only some species and genera performing three or more replications were able to ferment proteins, on the basis of the available literature. The short time span of 6 h between the inoculum separating the inoculum from the sample subjected to metagenome analysis did not result sufficient to enrich the culture of sole proteolytic species, since other groups fermenting organic acids or even carbohydrates increased up to the threshold of three generations. The outcome of this experiment confirmed that, also in very stringent growth conditions, such as those attained providing a sole carbon source, cross-feeding occurs in gut bacteria.

A further part of the project complements metagenomics studies that are in progress. Whereas most of the studies focused only on microbiota through metagenomics approaches to describe the composition of the feces or of the gut content, we wanted to extend this view encompassing the chemical composition of the fecal samples. In particular, we studied the fecal composition of two cohorts of healthy subjects and CKD patients, in order to match these data with metagomic profiles. The study highlighted a wide inter-individual variability for most of the parameters that have been analyzed. Only ammonium and total carbohydrates were significantly different between the two cohorts, and the observed trends suggested a less efficient saccharolytic metabolism of the fecal microbiota from CKD patients. For the other parameters, the low numerosity of these experiments and the wide range of the data limited the statistical significance. The kinetic of production or degradation of indole and p-cresole were determined using fresh microbiota from healthy volunteers, indicating tha p-cresol is a recalcitrant molecule that, mostly in presence of the precursor tyrosine, is produced, whereas it is hardly degraded. Conversely, indole, on the basis of its own presence or of availability the precursor tryprophan, is degraded or produced, respectively. In both cases, the diverse microbiota perform differently, confirming a different capability of each microbiota to carry out these reactions. This in agreement with the diverse compositions of the gut microbiota, which corresponds to functional differences that can affect the health status of each subject.

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8. Publications realized during the course of the PhD

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Research Article

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

Marta Simone,[1] Caterina Gozzoli,[1] Andrea Quartieri,[1] Giuseppe Mazzola,[2] Diana Di Gioia,[2] Alberto Amaretti,[1] Stefano Raimondi,[1] and Maddalena Rossi[1]

[1] *Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, Viale G. Campi 183, 41125 Modena, Italy*
[2] *Dipartimento di Scienze Agrarie, Università di Bologna, Viale Fanin 44, 40127 Bologna, Italy*

Correspondence should be addressed to Maddalena Rossi; maddalena.rossi@unimore.it

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

1. Introduction

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

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

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

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

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

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

2. Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

3. Results

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

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

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

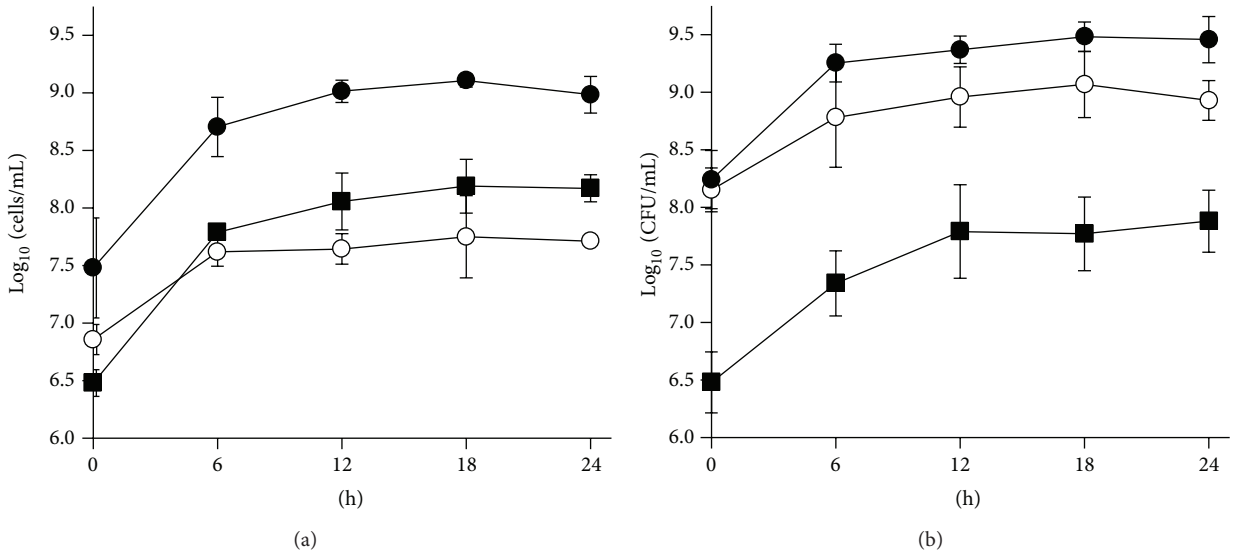

Figure 1: Time-course of total bacteria, *bifidobacteria*, and Enterobacteriaceae in cultures of infant gut microbiota. Eubacteria (●), *Bifidobacterium* (○), and Enterobacteriaceae (■) were quantified by FISH in control cultures (MC, (a)) and in cultures supplemented with *B. breve* B632 (PMC, (b)).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

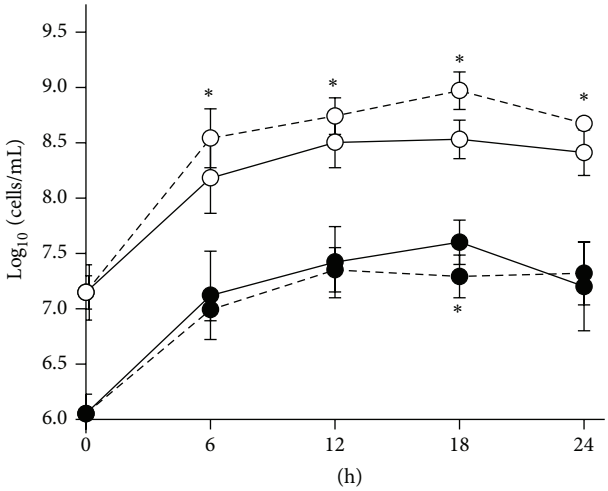

Figure 2: Time-course of *E. coli* and Enterobacteriaceae in cultures of infant gut microbiota. *E. coli* (●) and Enterobacteriaceae (○) were quantified by qPCR in control cultures (MC, dashed line) and in cultures supplemented with *B. breve* B632 (PMC, solid line). Data are means ± SD, $n = 4$. Stars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

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

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

4. Discussion

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

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

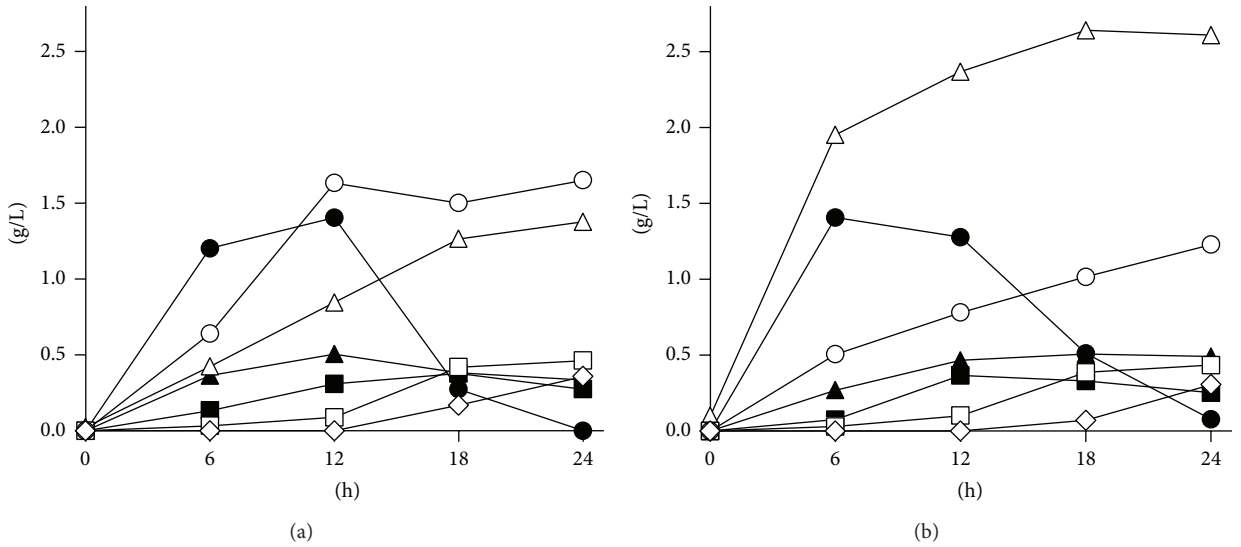

Figure 3: Time-course of fermentation products in cultures of infant gut microbiota. Ethanol (○), lactate (●), acetate (△), formate (▲), propionate (□), 2,3-butanediol (■), and butyrate (22C4) were determined in control cultures (MC, (a)) and in cultures supplemented with *B. breve* B632 (PMC, (b)).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

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

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

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

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

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

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

5. Conclusions

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

Conflict of Interests

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

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Comparison of culture-dependent and independent approaches to characterize fecal bifidobacteria and lactobacilli

Andrea Quartieri [a], Marta Simone [a], Caterina Gozzoli [a], Mina Popovic [a], Giuseppe D'Auria [b], Alberto Amaretti [a], Stefano Raimondi [a], Maddalena Rossi [a, *]

[a] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
[b] Genómica y Salud, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana, Valencia, Spain

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ABSTRACT

Different culture-dependent and independent methods were applied to investigate the population of bifidobacteria and lactobacilli in the feces of five healthy subjects. Bacteria were isolated on MRS, a complex medium supporting growth of lactobacilli and bifidobacteria, and on three selective media for bifidobacteria and two for lactobacilli. Taxonomic characterization of the isolates was carried out by RAPD-PCR and partial 16S sequencing. The selectivity of genus-specific media was also investigated by challenging colonies from MRS plates to grow onto each medium. In parallel, a quantitative and qualitative description of bifidobacteria and lactic acid bacteria was obtained by FISH, qPCR, TRFLP, and 16S rRNA gene sequencing.

Bifidobacteria did not fail to grow on their specific media and were easily isolated and enumerated, showing comparable quantitative data among culture-dependent and -independent techniques. The *Bifidobacterium* species identified on plates and those extracted from TRFLP and 16S rRNA gene sequencing were mostly overlapping.

Selective media for lactobacilli gave unsuitable results, being too stringent or too permissive. The quantification of lactobacilli through selective plates, qPCR, FISH, and 16S rRNA gene sequencing gave unreliable results. Therefore, unlike bifidobacteria, intestinal lactobacilli are still problematic in terms of quantification and accurate profiling at level of species and possibly of strains by both culture-dependent and culture-independent techniques.

1. Introduction

The mammalian gut is colonized by a complex microbial community composed largely of bacteria, whose numbers exceed 10^{11} cells per gram of intestinal content, belonging to over 1000 species, based on a recent review describing culturable bacteria isolated from feces [1]. Among the natural colonizers of the gut, bifidobacteria and lactobacilli benefit the host through a variety of different mechanisms, and specifically selected strains are claimed as probiotics [2,3].

Much literature has accumulated over the years on the development and utilization of selective cultural media to isolate and enumerate bifidobacteria and lactobacilli from a variety of matrices, such as dairy foods, probiotic-based products, and feces [4–7]. The specificity of some *Bifidobacterium*-selective media, including the commercial Bifidus Selective Medium (BSM), rests on the presence of antibiotics, such as mupirocin, nalidixic acid, neomycin sulphate, norfloxacin, or paromycin [8–11]. Other media, such as Raffinose Bifidobacterium (RB), owe their selectivity to a specific carbon source and to the presence of propionate and lithium chloride as inhibitory agents [12]. The selectivity of LAMVAB for fecal lactobacilli is based on vancomycin, low pH, and the characteristic blue or green color of the colonies resulting from bromocresol color transition [13]. LBS medium (*Lactobacillus* Selective) contains acetate/acetic acid to hinder growth of streptococci and molds, and is buffered at low pH to favor lactobacilli [14].

With the advent of high-throughput sequencing technologies, 16S rRNA gene sequencing has been applied to the investigation of gut microbiota, disclosing the different taxa colonizing the gut and shedding light on the ecology of such a complex environment. Even

* Corresponding author.
E-mail address: maddalena.rossi@unimore.it (M. Rossi).

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though metataxonomic information on the composition of intestinal microbiota is increasingly available, scarce attention has been focused on mining the abundance of specific microbial groups, such as bifidobacteria and lactobacilli, and to compare the results with those obtained by FISH (Fluorescent *in situ* Hybridization), qPCR (Quantitative Polymerase Chain Reaction), or plating on selective media [15].

FISH and qPCR, providing data mostly at genus level, are the techniques most frequently used to obtain quantitative information on bifidobacteria or lactobacilli [16–19]. The identification and quantification of *Bifidobacterium* and *Lactobacillus* species, and especially the traceability of specific strains or biotypes, still rely on methods that exploit cultivation on selective media, followed by taxonomic characterization [20,21]. TRFLP (Terminal Restriction Fragment Length Polymorphism) analysis could be utilized for quantification of the species, but a comprehensive database is still not available for fecal *Lactobacillus* species, whereas it has been developed for bifidobacteria [22].

The aim of this study was to compare methods providing information on bifidobacteria and lactobacilli of fecal human samples. Isolation and enumeration on selective media, followed by the taxonomic characterization of the isolates by RAPD-PCR and partial 16S sequencing, were carried out. In parallel, a quantitative description was obtained by 16S rRNA gene sequencing, FISH, qPCR, and TRFLP.

2. Materials and methods

2.1. Specimens and chemicals

The fecal specimens that were utilized for bacterial enumeration experiments were collected after obtaining written informed consent from five healthy volunteers (men, aged 20 to 40) who had not been treated with prebiotics and/or probiotics for one month, and antibiotics for at least three months. All the chemicals were supplied by Sigma (Stenheim, Germany), unless otherwise stated.

2.2. Culture-dependent enumeration of bifidobacteria and LAB

Fresh feces were homogenized (10% w/v) and serially diluted in isotonic buffered peptone water (BPW, Fluka–Sigma) supplemented with 0.5 g/L L-cysteine · HCl, then spread onto plates (10^{-4} to 10^{-9} dilutions). Lactobacilli MRS agar (BD Difco, Sparks, USA) supplemented with 0.5 g/L L-cysteine · HCl (hereinafter referred to as MRS) was utilized for both lactobacilli and bifidobacteria. BSM (Bifidus Selective Medium Agar, Fluka–Sigma), MRS supplemented with 50 mg/L mupirocin (hereinafter referred to as MUP) [10], and RB agar [13] were utilized to select bifidobacteria. LBS agar (BD Difco) and LAMVAB [14] were utilized to isolate fecal lactobacilli. The plates were incubated for 48 h at 37 °C in anaerobic cabinet under a 85% N_2, 10% CO_2, 5% H_2 atmosphere.

For each subject, 50 colonies from each of the *Bifidobacterium*-selective media were subjected to *Bifidobacterium*-specific PCR, utilizing the 16S rRNA gene primers Bif164/Bif662 according to literature [23]. In order to validate the selectivity of *Lactobacillus*-selective media, 100 colonies per subject per medium, obtained by direct plating in LBS and LAMVAB, were randomly picked, clustered through RAPD-PCR analysis, and classified by 16S partial sequencing. Furthermore, for each subject, 200 colonies were randomly picked from MRS plates at the lowest dilutions giving single colonies, and were seeded onto RB, BSM, MUP, LBS, and LAMVAB plates. The bacteria grown on the MRS plates at the dilution of 10^{-5} were replica-plated onto LBS and LAMVAB plates. All the colonies isolated by replica plating were taxonomically characterized through RAPD-PCR clustering and 16S partial sequencing.

2.3. Taxonomic attribution of bacterial isolates

The gDNA was extracted from bacterial colonies using Instagene matrix (Bio-Rad) and was subjected to RAPD-PCR amplification with M13-RAPD primer (5′-GAGGGTGGCGGTTCT-3′). The reaction was performed in 15 μL of DreamTaq Buffer, containing 50 ng of template gDNA from the isolates, 7.5 pmol of primer, 1.5 nmol of each dNTP, and 0.75 U of DreamTaq polymerase. The thermocycle was the following: 94 °C for 4 min; 45 cycles at 94 °C for 1 min, 34 °C for 1 min, and 72 °C for 2 min; 72 °C for 7 min. The PCR products were electrophoresed for 4 h at 160 V in a 25 × 25 cm 2% (w/v) agarose gel in TAE buffer. RAPD-PCR fingerprints were digitally captured and were analyzed with Gene Directory 2.0 (Syngene, UK) software, which calculated similarities and derived a dendrogram with an unweighted pair group method with arithmetic means (UPGMA).

To attribute each biotype to a species, a portion of 16S rDNA was amplified with the universal primers (forward primer: 59-TGGAGAGTTTGATCCTGGCTCAG-39; reverse primer: 59-TACCG CGGCTGCTGGCAC-39) spanning positions 5–532 (inclusive) of *Escherichia coli* K-12 (GenBank accession no. NC_000913) [24], sequenced, and compared with Genbank database. Amplification was performed in 50 μL of PCR Master Mix (Thermo Fisher Scientific), containing 10 pmol of each primer, and 50 ng of gDNA. The thermocycle was the following: 94 °C for 4 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 72 °C for 7 min.

2.4. Fluorescence in situ hybridization (FISH)

Bacteria belonging to *Bifidobacterium* genus and *Lactobacillus-Enterococcus* group were quantified by FISH, using the probes Bif164 (5′-CATCCGGCATTACCACCC-3′) and Lab158 (5′-GGTATTAGCAYCTGTTTCCA-3′), respectively [16,25]. The species of human intestinal lactobacilli and enterococci potentially recognized by FISH probe Lab158 according to SILVA database (http://www.arb-silva.de) are presented in Table 1. Feces were suspended (10% w/v) in PBS buffer, homogenized, and gently centrifuged to remove the solids (300 ×g for 1 min). The supernatant was diluted 1:4 with 40 g/L paraformaldehyde and kept at 4 °C for 16 h. The cells, properly diluted with PBS (0–1000 fold), were applied onto gelatin-coated slides and were dehydrated with ethanol. For the enumeration of *Lactobacillus-Enterococcus* group, the cells were treated with 1 mg/ml lysozyme for 15 min at 37 °C. Dehydrated cells were covered with 100 μL of hybridization buffer (20 mM TRIS-HCl, 0.9 M NaCl, 1 g/L SDS) containing 0.75 μM FITC-labeled probe and were kept for 16 h at 53 °C in a dark and moisture-saturated chamber. Hybridized slides were washed with 20 mM TRIS-HCl and 0.9 M NaCl, and were covered with Vectashield (Vector Laboratories, Burlingame, CA). Fluorescent cells were counted with an epifluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan). Depending on the number of fluorescent cells, 30 to 100 microscopic fields were counted and averaged in each slide. Each sample was enumerated in triplicate.

2.5. qPCR

Quantification of *Bifidobacterium* and *Lactobacillus* group was achieved with primer pairs BiTOT-F/BiTOT-R (TCGCGTCYGGTGTGAAAG/CCACATCCAGCRTCCAC) and Lac-F/Lac-R (GCAGCAGTAGGGAATCTTCCA/GCATTYCACCGCTACACATG), respectively [17,26]. The species of human intestinal lactobacilli potentially recognized by qPCR primers Lac-F/Lac-R are presented in Table 1. Reactions were

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Table 1
Species of the human intestinal lactobacilli and enterococci potentially recognized by FISH probe Lab158 and qPCR primers Lac-F/Lac-R, according to SILVA database.

Species	FISH Lab158	qPCR Lac-F/Lac-R
Enterococcus		
E. casseliflavus	+	−
E. faecalis	+	−
E. faecium	+	−
E. gallinarum	+	−
E. hirae	+	−
Lactobacillus		
L. acidophilus	+	+
L. amylolyticus	+	+
L. antri	+	+
L. brevis	+	+
L. buchneri	+	+
L. casei	+	+
L. colehominis	+	+
L. crispatus	+	+
L. delbruecki	+	+
L. fermentum	+	+
L. gasseri	+	+
L. helveticus	+	+
L. hilgardii	+	+
L. iners	+	+
L. jensenii	+	+
L. johnsonii	+	+
L. paracasei	+	+
L. plantarum	+	+
L. reuteri	+	+
L. rhamnosus	+	+
L. sakei	+	+
L. salivarius	+	+
L. ultunensis	+	+
L. vaginalis	+	+

performed in 20 μL of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, CA, USA), containing 2.5 ng of template gDNA, and 6 pmol of each primer. The following thermocycle was set in a CFX96 Real-Time System (Bio-Rad): 98 °C for 2 min; 40 cycles of 98 °C for 5 s, 60 °C for 5 s, and 95 °C for 1 min; 65 °C for 1 min. To control the specificity of amplification, analysis of product melting curve was performed at the end of the reaction.

The data obtained were converted according with the rRNA copy number available at the rRNA copy number database, using the mean copy number of 3.57 for *Bifidobacterium* and 5.71 for *Lactobacillus* spp [27,28].

2.6. TRFLP analysis of bifidobacteria

The gDNA from fecal samples underwent a PCR reaction with the primers NBIF389/NBIF1018REV (5′-[HEX]-GCCTTCGG GTTGTAAAC-3′/5′-GACCATGCACCACCTGTG-3′). Reactions took place in 50 μL of DreamTaq Green Buffer (Thermo Fisher Scientific, Waltham, MA, USA), containing 50 ng of gDNA, 5 pmol of each primer, 2.5 nmol of each dNTP, and 2.0 U of DreamTaq polymerase (Thermo Fisher Scientific). The following thermocycle was utilized: 95 °C for 5 min; 30 cycles 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min; 72 °C for 5 min. Amplification was confirmed by gel electrophoresis, then DNA was purified using NucleoSpin Gel and PCR Clean-up purification kit (Macherey–Nagel, Germany), and quantified with NanoPhotometer P-Class (Implen GmbH, Munchen, Germany). 10 μL of DNA was digested with 1 U/μL *Alu*I and *Hae*III (Bio-Rad) in separate reactions. After 3 h at 37 °C, the enzymes were heat inactivated according to supplier's instructions. 1.5 μL of properly diluted digestion mixture was used for fragment analysis on an ABI 3130 Capillary Electrophoresis Genetic Analyzer (Applied Biosystems/Thermo Fisher Scientific), using the ROX 50–500 (Gel Company, San Francisco, CA) size marker. The electropherograms were read using Peak Scanner software v1.0, (Applied Biosystems). Assignment of size standard peaks was performed, then the results were compared with the empirical database developed by Lewis et al. [22], which included the following *Bifidobacterium* species: *B. adolescentis*, *B. animalis*, *B. bifidum*,*B. breve*, *B. longum* group, and *B. pseudocatenulatum*.

2.7. 16S rRNA gene sequencing

The genomic DNA (gDNA) from fecal samples was extracted with QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany), quantified with Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and adjusted to the concentration of 5 ng/μL.

Amplicons of approximately 460 bp were generated using primers targeting V3 and V4 regions of 16S rDNA and bearing Illumina overhang adapters [29]. The initial PCR reactions were performed with the following program: 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 7 min at 72 °C. A step of post-PCR cleanup from free primers and primer dimers was performed with AMPure XP beads (Beckman Coulter, Pasadena, CA, USA). Illumina Nextera XT Index kit (Illumina, San Diego, CA, USA) with dual 8-base indices was used to allow for multiplexing. Following the Nextera dual-indexing strategy, two unique indices located on either end of the amplicon were chosen. To incorporate the indices to 16S amplicons, PCR reactions were performed with the following program: 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min. Barcoded libraries were cleaned with AMPure XP beads, quantified with Qubit Fluorimeter, pooled at the normalized concentration of 4 nM each, and sequenced by 2 × 300 bp paired-end sequencing on the MiSeq platform using MiSeq v3 Reagent Kit (Illumina). Each run included a minimum of 5% PhiX to serve as an internal control for those low-diversity libraries.

Mate pairs read were joined using “fastq-join” program from “ea-tools” suite (http://code.google.com/p/ea-utils) [30]. Quality assessment was performed by the use of “prinseq-lite” program removing sequences shorter than 200 nucleotides and applying 5′ trimming maintaining at least a mean quality score of 20 in a window of 20 bp [31]. Eventual chimeric sequences formed during PCR reactions have been eliminated using “usearch” program applying default parameters [32]. Taxonomic assignation has been obtained using “RDP_classifier” trained with GreenGene otus dataset (gg_otus_99, v13_05) by the use of Qiime suite [33–35]. Diversity and statistics analysis have been carried out by the use of R environment from the R-core team (http://www.R-project.org).

2.8. Statistical analysis

Bacterial enumerations are means of three separate experiments. Means were compared applying Student's t test to total counts of cfu/g or cells/g, and were considered statistically significant for $P < 0.05$.

2.9. Accession numbers

The partial sequences of 16S rDNA, utilized for the attribution of the new isolates, were deposited at GenBank, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nucleotide), with accession numbers from KM457440 to KM457482. Illumina sequences were deposited at European Nucleotide Archive (https://www.ebi.ac.uk/ena) with accession PRJEB9917.

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3. Results

3.1. Quantification of bifidobacteria and LAB by culture-dependent and -independent approaches

Bifidobacteria, lactobacilli, and/or LAB were quantified in the feces of 5 healthy volunteers through culture-dependent approaches (Table 2). Plate counts on MRS ranged from 8.82 to 9.21 $\log_{10}$ cfu/g. For each sample, *Bifidobacterium* selective plates (RB, BSM, and MUP) yielded similar viable counts ($P > 0.05$), from 8.51 to 9.15 $\log_{10}$ cfu/g. All the assayed colonies from RB, BSM, and MUP were positive to *Bifidobacterium*-specific PCR. *Lactobacillus*-selective plates (LBS and LAMVAB) gave counts much lower than RB, BSM, and MUP ($P < 0.05$). In subjects V2 and V4, LBS and LAMVAB counts were in good agreement ($P > 0.05$), while LAMVAB counts in subjects V1, V3, and V5 were significantly lower ($P < 0.05$).

FISH and qPCR enumeration of bifidobacteria gave consistent results, ranging from 8.53 to 9.68 $\log_{10}$ cells/g (Table 3), in the same magnitudes of plates counts. FISH targeting the genera *Lactobacillus* and *Enterococcus* gave counts ranging from 7.53 to 7.94 $\log_{10}$ cells/g, while qPCR targeting the sole *Lactobacillus* genus gave significantly lower counts ($P < 0.05$), ranging from 6.27 to 6.98 $\log_{10}$ cells/g.

3.2. Species profiling of MRS isolates

Resting on the assumption that both bifidobacteria and lactobacilli can grow on MRS, 200 colonies from MRS plates were randomly selected, subjected to RAPD-PCR clustering, and each biotype was taxonomically characterized through 16S partial sequencing (Table 4).

Most of the isolates were bifidobacteria (908/1000), attributed to the species *B. breve* and *B. longum*, with *B. longum* comprising the biggest proportion (870/908). All the 200 colonies from sample V5 were ascribed to five different biotypes of *B. longum*. In the other samples, isolates other than bifidobacteria were mainly LAB (79/92), the vast majority (66/79) belonging to the species *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus hirae*. Enterococci were particularly abundant in MRS plates of subject V2 (49/200). Lactobacilli were found only in the samples V2 and V3, where *L. paracasei* and *L. mucosae* biotypes were identified, respectively.

3.3. Species profiling of LBS and LAMVAB isolates

In order to validate the selectivity of the media, 100 colonies per subject, obtained by direct plating in LBS and LAMVAB, were randomly picked, clustered through RAPD-PCR analysis, and classified by 16S partial sequencing (Table 5). From subject V1, only enterococci were isolated on LBS plates, whereas no colonies were present in LAMVAB (<4 $\log_{10}$ cfu/g). In the other subjects, the same biotypes of *Lactobacillus* were generally obtained in LBS and LAMVAB, with few exceptions.

From sample V2, 5 biotypes belonging to the species *L. rhamnosus*, *L. parabuchneri*, and *L. paracasei* were identified. In V3, *L. mucosae* was isolated in both LBS and LAMVAB, and represented the majority of the isolates, while *L. gasseri* was found only on LBS. In subject V4, the most represented biotypes were *L. oris* V4-09 and *L. gasseri* V4-06, followed by other LAB belonging to the species *E. faecium*, *L. gasseri*, *L. oris*, and *L. salivarius*. Two minor biotypes of *L. gasseri* (V4-07 and V4-08) were found only in LBS, and *L. salivarius* V4-12 only in LAMVAB. Subject V5 presented two biotypes of *L. gasseri* and *L. rhamnosus*, the former being the most abundant on both media.

In order to verify whether direct plating onto LBS and LAMVAB hampered growth of fecal lactobacilli, properly diluted fecal samples were firstly allowed to grow onto MRS plates, then colonies were replica-plated on LBS and LAMVAB. Counts on replica-plates were similar with those obtained by the direct counting. Compared with direct counts on *Lactobacillus*-selective media, the same bacterial biotypes were in general obtained by replica-plating on LBS and LAMVAB of bacteria previously grown onto MRS plates. The sole exceptions were two biotypes of *L. paracasei* and one of *L. frumenti* (from subjects V2 and V4, respectively) that were found on LAMVAB replicated plates, but were not retrieved through the direct count in this medium.

3.4. Media selectivity

The isolates recovered from MRS plates and taxonomically characterized were challenged for growth onto the selective plates of RB, BSM, MUP, LBS, and LAMVAB, in order to compare the selectivity of these media and to evaluate whether certain bifidobacteria or LAB escaped the direct count onto *Bifidobacterium*-selective and *Lactobacillus*-selective plates. Remarkably, in some cases, isolates belonging to the same biotype did not behave homogeneously with respect to growth on different media (Table 4).

All the bifidobacteria grew on RB, BSM, and MUP, and some isolates also on LBS and/or LAMVAB. For instance, all the *Bifidobacterium* biotypes retrieved from sample V3 grew in LBS. Similarly, most of the bifidobacteria obtained from subject V5 grew in LBS, and some of them exhibited growth in LAMVAB as well.

All the enterococci grew onto BSM, most on RB, whereas only MUP inhibited their growth. Among *Lactobacillus*-selective media, LAMVAB hindered growth of all the enterococci, whereas LBS permitted the growth of some biotypes. *L. paracasei* V02-05 grew onto all the media putatively selective for bifidobacteria, while *L. mucosae* V03-04 was inhibited on MUP.

Table 2
Bacterial viable counts (reported as $\log_{10}$ cfu/g) of the feces of five subjects, measured with plates of MRS, *Bifidobacterium*-selective, and *Lactobacillus*-selective media[a].

Subject	MRS	*Bifidobacterium*-selective			*Lactobacillus*-selective	
		RB	BSM	MUP	LBS	LAMVAB
V1	8.85	8.69	8.72	8.51	7.75	<4*
V2	8.82	8.70	8.70	8.63	6.25	6.37
V3	9.11	8.87	8.80	8.97	7.59	7.10*
V4	9.21	9.02	9.05	9.15	6.38	5.98
V5	8.96	8.76	8.83	8.93	5.85	4.18*

Within each group of selective media, stars indicate statistically different means ($P < 0.05$).
[a] Values are means, n = 3, SD always <0.3 $\log_{10}$ cfu/g.

Table 3
Concentration of bifidobacteria and LAB (reported as $\log_{10}$ cells/g) in the feces of five volunteers, measured with FISH and qPCR[a].

Subject	*Bifidobacterium*		LAB	
	FISH	qPCR	FISH	qPCR
V1	8.83	9.30*	7.64	6.82*
V2	8.53	9.13*	7.53	6.98*
V3	9.01	9.41*	7.94	6.91*
V4	9.30	9.68*	7.90	6.27*
V5	9.18	9.20	7.87	6.53*

Stars indicate statistically significant difference between techniques means ($P < 0.05$).
[a] Values are means, n = 3, SD always <0.2 $\log_{10}$ cells/g.

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Table 4
Bacterial biotypes identified within 200 colonies per subject, randomly picked from MRS plates. Growth on RB, BSM, MUP, LBS, and LAMVAB plates is reported.

Subject	Accession number	Attribution	No. of colonies		RB	BSM	MUP	LBS	LAMVAB
V1	KM457440	*B. longum* V1-01	196	191	+	+	+	−	−
				5	+	+	+	+	−
	KM457440	*E. faecalis* V1-02	4	4	+	+	+	−	−
V2	KM457445	*B. longum* V2-01	149	127	+	+	+	−	−
				22	+	+	+	+	−
	KM457446	*E. hirae* V2-02	45	18	+	+	−	+	−
				18	+	+	−	−	−
				9	−	+	−	+	−
	KM457447	*E. hirae* V2-03	3	3	+	+	−	+	−
	KM457448	*E. faecium* V2-04	1	1	+	+	−	+	−
	KM457449	*L. paracasei* V2-05	2	2	+	+	+	+	+
V3	KM457456	*B. longum* V3-01	122	122	+	+	+	+	−
	KM457457	*B. breve* V3-02	38	21	+	+	+	−	−
				17	+	+	+	+	−
	KM457458	*S. hominis* V3-03	13	13	+	−	−	−	−
	KM457459	*L. mucosae* V3-04	11	7	+	+	−	+	+
				4	+	+	−	−	+
	KM457460	*B. longum* V3-05	10	5	+	+	+	+	−
				5	+	+	+	−	−
	KM457461	*E. hirae* V3-06	5	5	+	+	−	−	−
	KM457462	*E. faecium* V3-07	1	1	+	+	−	−	−
V4	KM457464	*B. longum* V4-01	104	95	+	+	+	−	−
				9	+	+	+	+	−
	KM457465	*B. longum* V4-02	77	64	+	+	+	−	−
				13	+	+	+	+	−
	KM457466	*B. longum* V4-03	12	9	+	+	+	−	−
				3	+	+	+	+	−
	KM457467	*E. faecium* V4-04	7	5	+	+	−	+	−
				2	+	+	+	−	−
V5	KM457476	*B. longum* V5-01	139	66	+	+	+	+	−
				51	+	+	+	−	−
				22	+	+	+	+	+
	KM457477	*B. longum* V5-02	21	21	+	+	+	+	−
	KM457478	*B. longum* V5-03	11	6	+	+	+	+	−
				5	+	+	+	−	+
	KM457479	*B. longum* V5-04	20	20	+	+	+	−	−
	KM457480	*B. longum* V5-05	9	5	+	+	+	−	+
				4	+	+	+	+	−

Table 5
Bacterial biotypes capable of growth on LBS and LAMVAB plates. 50 colonies per subject and per medium were randomly selected from direct plating (DP) and from replica plating (RP) of bacteria previously grown onto MRS plates.

Subject	Accession Number	Attribution	LBS		LAMVAB	
			DP	RP	DP	RP
V1	KM457442	*E. hirae* V1-03	24	29	0	0
	KM457443	*E. hirae* V1-04	20	18	0	0
	KM457444	*E. faecium* V1-05	6	3	0	0
V2	KM457450	*L. rhamnosus* V2-06	21	11	23	16
	KM457449	*L. paracasei* V2-05	5	15	4	11
	KM457451	*L. paracasei* V2-07	19	17	14	12
	KM457452	*L. parabuchneri* V2-08	3	5	7	7
	KM457453	*L. rhamnosus* V2-09	2	2	2	0
	KM457454	*L. paracasei* V2-10	0	0	0	2
	KM457455	*L. paracasei* V2-11	0	0	0	2
V3	KM457459	*L. mucosae* V3-04	40	48	50	50
	KM457463	*L. gasseri* V3-08	10	2	0	0
V4	KM457465	*B. longum* V4-02	0	4	0	0
	KM457467	*E. faecium* V4-04	4	10	0	0
	KM457468	*L. frumenti* V4-05	0	0	0	3
	KM457469	*L. gasseri* V4-06	13	8	34	15
	KM457470	*L. gasseri* V4-07	1	5	0	0
	KM457471	*L. gasseri* V4-08	1	0	0	0
	KM457472	*L. oris* V4-09	24	12	7	22
	KM457473	*L. oris* V4-10	1	0	2	0
	KM457474	*L. salivarius* V4-11	6	11	5	10
	KM457475	*L. salivarius* V4-12	0	0	2	0
V5	KM457481	*L. gasseri* V5-06	47	50	38	44
	KM457482	*L. rhamnosus* V5-07	3	0	12	6

3.5. TRFLP analysis of bifidobacteria

In the fecal samples of all the volunteers, TRFLP revealed four different species or groups: *B. adolescentis*, *B. bifidum*, *B. longum* group, and *B. pseudocatenulatum* (Fig. 1), with *B. longum* group and

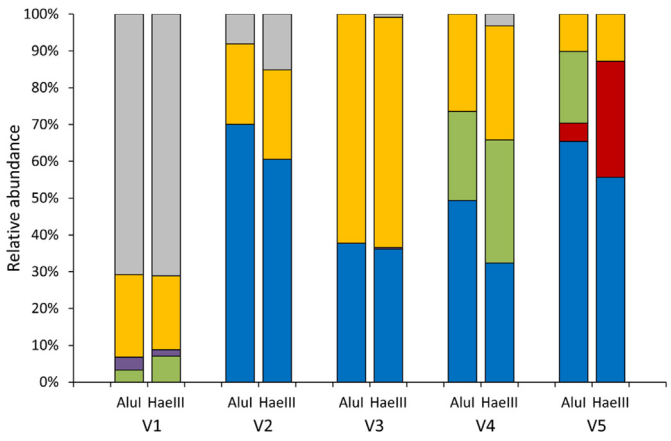

Fig. 1. Relative abundance of *Bifidobacterium* species, determined by TRFLP. Percentages of the total post-filtering peak area from the electropherograms, excluding primer-dimer and smaller peaks (<35 bp), are reported. *B. adolescentis*, blue; *B. bifidum*, red; *B. longum* group, yellow; *B. pseudocatenulatum*, green; *B. pseucodcatenulatum*/*B. bifidum*, violet; *Bifidobacterium* sp., grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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B. adolescentis being the most represented in terms of relative abundance among the bifidobacterial community. *B. longum* was found in all the samples, ranging from 11 to 63%, while *B. adolescentis* was not identified only in subject V1, while in the others its abundance ranged from 32 to 70%. *B. pseudocatenulatum* and *B. bifidum* occurred in lower abundance and not in all the samples. In subject V5, *B. pseudocatenulatum* was found only with *Alu*I restriction, whereas both the enzymes detected the presence of *B. bifidum*, but *Hae*III indicated a higher amount than *Alu*I. Some terminal restriction fragments (TRFs) were not taxonomically attributed with certainty at species level. In sample V1, TRFs consistent with both *B. pseudocatenulatum* and *B. bifidum* were generated with both the enzymes. Un-attributed *Bifidobacterium* species was assessed by the detection of TRFs that did not fit any species in the empirical database. They were a minority in most of the samples, whereas at least 3 undefined species accounted for approx. the 70% of the TRFs obtained in subject V1.

3.6. 16S rRNA gene sequencing

16S rRNA gene sequencing was employed to obtain a comprehensive overview of the gut microbiota of 4 out of the 5 healthy volunteers (V3 fecal sample was not available for this analysis), from which the information on the relative abundance of bifidobacteria and lactobacilli was extracted. The microbiota of all the subjects was dominated by the phyla *Bacteroidetes* (41.8–59.3%) and *Firmicutes* (38.6–54.1%), followed by *Proteobacteria* (1.1–3.2%) and *Actinobacteria* (0.3–1.8%) (Table 6). Among *Firmicutes*, reads attributed to the class *Bacilli* ranged between 0.1 and 2%, with *Lactobacillales* accounting for 26.4–97.8% of *Bacilli*. In all the samples, most of the reads of *Lactobacillales* belonged to the genera *Enterococcus*, *Lactobacillus*, and *Streptococcus*, with *Streptococcus* being the most represented genus (77.0–88.5%), including members of species *S. alactolytus* (in samples V4 and V5), *S. anginosus* (in samples V1 and V5), and *S. infantis* (all samples) followed by *Lactobacillus* (2.7–20.5%), and by *Enterococcus* (1–5%) with one identified species, *Enterococcus casseliflavus*. In all the samples, the reads of the genus *Lactobacillus* were 2.5–20-fold more numerous than the ones of *Enterococcus*. In subjects V1, V2, and V5 *Actinobacteria* were dominated by *Bifidobacteriales*, accounting for 80–91%. In subject V4, *Coriobacteriales* were more abundant than *Bifidobacteriales* (69 and 29%, respectively). In all the samples, most *Bifidobacteriales* belonged to the genus *Bifidobacterium*, that accounted for 0.01–2% of total bacteria. The species *B. adolescentis* (all samples), *B. bifidum* (subjects V2 and V5), and *B. longum* (all samples) were identified. The genus *Bifidobacterium* was more represented than the genus *Lactobacillus* in all of the samples, with a ratio of 138, 90.4, 2.7, and 49.9 in samples V1, V2, V4, and V5, respectively.

Table 6
Percentage of Illumina reads attributed to the main intestinal phyla, to the orders *Bifidobacteriales* and *Lactobacillales*, and to the genera *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Streptococcus*.

Phylum/Order/Genus	V1	V2	V4	V5
Actinobacteria	1.259	0.398	0.339	1.773
Bifidobacteriales	1.125	0.318	0.097	1.609
Bifidobacterium	1.124	0.318	0.097	1.609
Firmicutes	54.099	38.552	46.313	46.949
Lactobacillales	0.053	0.128	0.175	0.178
Lactobacillus	0.008	0.004	0.038	0.032
Enterococcus	0.003	0.001	0.002	0.002
Streptococcus	0.041	0.113	0.134	0.137
Bacteroidetes	41.807	59.253	52.092	48.088
Proteobacteria	2.779	1.706	1.062	3.153
Other phyla	0.060	0.090	0.194	0.048

4. Discussion

This study aimed to compare different culture-dependent and -independent approaches to qualitatively and quantitatively describe bifidobacteria and lactobacilli of human fecal samples, and to investigate the suitability of selective media for their enumeration and isolation.

BSM, MUP, and RB, claimed as selective media for bifidobacteria [9,12], yielded comparable recovery, with concentrations laying on the same magnitude orders of FISH and qPCR. However, qPCR counts were generally higher than FISH ones ($P < 0.05$), likely because dead or metabolically inactive cells were not detected by FISH, due to low rRNA content [36]. The appropriateness of each medium for bifidobacteria was proved by the successful genus-specific PCR amplification of the tested isolates and the good agreement among plate counting and molecular methods.

Unlike bifidobacteria, enumeration of fecal lactobacilli by cultural and molecular methods was not reliable. Correspondence between data obtained by selective plates, qPCR, FISH, and 16S rRNA gene sequencing resulted inconsistent and unpredictable. Discrepancies between FISH and qPCR can be due to the different pattern of species recognized (Table 1). In particular probe Lab158 hybridizes the rRNA of *E. faecalis*, *E. faecium,* and *E. hirae* which can represent important components of intestinal LAB, whereas qPCR primers exclude these species. However, based on metaxonomic analysis, lactobacilli outnumbered enterococci by 2.5–20 times, confirming inconsistency among the results achieved through the diverse technologies. Further discrepancies were detected in the ratio of lactobacilli and bifidobacteria observed by 16S rRNA gene sequencing and counted by plating. Unfortunately, 16S rRNA gene sequencing does not provide quantitative data, and it is hard to discriminate between its bias and the issues of lactobacillus selective media.

Counts on LBS and LAMVAB were significantly different in 3 out of 5 samples (V1, V3, and V5; $P < 0.05$), with up to two magnitudes of difference. In particular, sample V1 yielded 7.75 $\log_{10}$ cfu/g in LBS, whereas no colonies (<4 $\log_{10}$ cfu/g) were recovered in LAMVAB.

Diverse *Enterococcus* biotypes were recovered onto LBS from subjects V1 and V4. Albeit LAMVAB hindered growth of enterococci, it is plausible that it restricted also growth of some strains belonging to the 38 *Lactobacillus* species isolated from the human intestinal microbiota [37]. In fact, vancomycin susceptibility of lactobacilli is greatly variable among different species and even within the same species, with MIC ranging from 1 to >256 μg/ml [38].

While lactobacilli in food matrices (e.g. dairy products, meat, and vegetables) can be easily retrieved with MRS plates [39,40], isolation of fecal lactobacilli is hampered by bifidobacteria, that present similar nutritional and environmental requirements and are much more numerous. In this study, in order to evaluate the suitability of selective media and the capability of fecal bifidobacteria and LAB to grow on restrictive media, 1000 strains recovered from MRS plates were challenged for growth in RB, BSM, MUP, LBS, and LAMVAB (Table 4).

As expected, the counts on MRS were close to those on *Bifidobacterium*-selective media and most of the isolates were bifidobacteria. The majority of the LAB failed to grow on MUP plates, whereas they were unpredictably capable of growth in RB and/or BSM. Most of bifidobacteria grew only on *Bifidobacterium*-selective media, growth in LAMVAB was rare, whereas LBS supported growth of the 17.5% of the bifidobacterial isolates. As a whole, both LBS and LAMVAB resulted not suitable for quantification and isolation of intestinal lactobacilli because the former may be too permissive,

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and the latter too stringent.

Description of bifidobacteria at species level obtained by 16S sequencing of MRS isolates, TRFLP, and16S metataxonome analysis were compared. Only *B. longum* was isolated in MRS from all the samples, whereas *B. breve* was found only in sample V3. TRFLP and 16S rRNA gene sequencing confirmed that *B. longum* was present in all the samples. Both culture-independent techniques revealed that *B. adolescentis* represented a major component in subjects V2, V4, and V5, albeit no biotypes belonging to this species were isolated from any sample. *B. pseudocatenulatum* and *B. bifidum*, identified by TRFLP, were not found among the isolates in MRS nor by 16S rRNA gene sequencing. The relatively limited number of *Bifidobacterium* colonies characterized (149–200 per sample) and the drawbacks of TRFLP, such as the pseudo-quantitative outcome, the low taxonomic resolution, the limited ability to detect multiple species with similar abundance, the difficult detection of low abundance species in presence of a heavily dominating one, may have led to partially inconsistent results [22]. However, we should consider the possibility that not all the bifidobacteria propagate at the same extent in selective media, and some species may not grow.

The high-throughput survey of 16S rRNA genes confirmed that bifidobacteria and LAB are minor components of fecal microbiota. Although the abundance of 16S PCR amplicons may be biased by several issues, including the different 16S rRNA gene copy number, and is not reproducible for low template concentrations [41,42], bacteria belonging to the genus *Bifidobacterium* outnumbered LAB up to two magnitudes, in agreement with FISH and qPCR results.

5. Conclusions

The present work provides a comparison of different approaches to enumerate bifidobacteria and lactobacilli in human fecal samples. It is based on only a small samples size, and these exploratory data could hopefully be validated by a larger cohort of samples.

Culture-independent techniques, such as 16S rRNA gene sequencing, FISH, RAPD-PCR, qPCR, and TRFLP, have been applied and the suitability of the most common culture media for enumeration, isolation, and traceability of these bacteria was explored.

For quantification of bifidobacteria, the three selective media and the culture-independent techniques assessed their efficacy by giving comparable results. The profiling of bifidobacterial population gave consistent results among the different techniques, although some discrepancies were observed.

On the contrary, lactobacilli proved to be still problematic in terms of accurate profiling by both culture-dependent and -independent techniques and efforts are still necessary to optimize molecular techniques and to formulate appropriate media.

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Review

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Potential Impact of Probiotic Consumption on the Bioactivity of Dietary Phytochemicals

Maddalena Rossi,* Alberto Amaretti, Alan Leonardi, Stefano Raimondi, Marta Simone, and Andrea Quartieri

Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy

ABSTRACT: Many healthy phytochemicals occur in food in the form of esters, glycoconjugates, or polymers, which are not directly bioavailable. Probiotic lactobacilli and bifidobacteria, which have evolved within the colonic ecosystem where indigestible oligo- and polysaccharides are their sole carbon sources, bear several glycosyl-hydrolases and can contribute to release the aglycones from glycoconjugated phytochemicals. Among the glycosyl-hydrolases, β-glucosidases are the most pertinent, because many phytochemicals are glucoconjugates. β-Glucosidase-positive probiotic bacteria were proved to release the aglycones of isoflavones and lignans in vitro, but studies in vivo are scarce. A positive correlation between probiotic consumption and urinary and/or plasma levels of isoflavone or lignan metabolites was not established. However, the strains used in the trials were not validated for the enzymatic properties or for the ability to hydrolyze lignans or isoflavones. Thus, activation of specific phytochemicals by probiotic bacteria still needs substantial efforts to be proved.

KEYWORDS: *phytochemicals, probiotics, Lactobacillus, Bifidobacterium, microbiota, isoflavones, lignans*

INTRODUCTION

Edible plants are dietary sources of hundreds of non-nutritional phytochemicals. Many phytochemicals exert a number of beneficial activities, including antioxidant, antitumoral, anti-inflammatory, and estrogenic-like properties, as demonstrated by numerous epidemiologic, clinical, and experimental studies.[1−3]

The level of bioactive phytochemicals within the body is largely determined by diverse phenomena, such as the digestive transformation of native compounds, absorption in the intestine, hepatic activity, and biliary or urinary excretion.[4,5] The phytochemicals that are not absorbed in the small intestine reach the colon, where they may undergo extensive biotransformation by the resident microbiota.[5−7] This bacterial transformation may lead to the inactivation and/or degradation of phytochemicals or may cause the production of compounds with enhanced biological activity or bioavailability. Examples of the specific conversion of diverse molecules into bioactive metabolites accomplished by the microbiota are the conversion of lignans into enterodiol and enterolactone (ED and EL, respectively) and the conversion of soy isoflavones into *S*-equol.[8−11] During the course of absorption, phytochemicals are conjugated in the intestine and later in the liver, being subjected to methylation, sulfation, and β-glucuronidation. Mammalian conjugates can be secreted in the duodenum with the bile as hydrophilic conjugates or can be effluxed from the enterocytes directly to the gut lumen. Furthermore, the colonic microbiota is involved in the enterohepatic recycling of phytochemicals. In fact, the microbial β-glucuronidase and sulfatases can deconjugate the excreted phytochemicals in the colon, where they can be reabsorbed, leading to a longer presence in the body.[5,12]

Bacterial transformations of phytochemicals that reach the colon modify their absorption and bioavailability. For instance, in vivo studies demonstrated that a diverse availability of bioactive compounds generally occurs among different subjects, this interindividual variation being mainly attributed to differences in the composition of the gut microbiota.[13−17] This review provides an update on current advances on the impact of probiotic consumption on metabolism and bioavailability of phytochemicals. It addresses the genetic and enzymatic features of probiotic bacteria potentially involved in the transformation of phytochemicals and the outcome of in vivo trials carried out with associations of probiotics and phytochemicals.

GUT MICROBIOTA AND PROBIOTICS

The human colon harbors one of the most diversified and densely populated bacterial ecosystems on Earth, dominated by anaerobic bacteria belonging to the phyla of Firmicutes (which include Clostridiales and Lactobacillales), Bacteroidetes, and Actinobacteria (which include Bifidobacteriales), in numbers exceeding 10^{11} cells/g of intestinal content.[11] The host and the commensal bacteria establish a mutualistic relationship, which has a major impact on the nutrition and overall health status of the host.[18,19] The colonic microbiota is maintained in a constant-temperature environment and is provided with a broad spectrum of substrates undigested and unabsorbed.[18,19] On the other hand, the microbiota offers to the host protection against infections, plays a role in the modulation of the immune system, and supplies carbon, energy, vitamins, and bacterial-activated dietary metabolites.[20,21]

For its energy needs, the bacterial community exploits unabsorbed oligo- and polysaccharides, proteins, and peptides. They are broken down by bacterial enzymes into their

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oligomeric and/or monomeric components, which are fermented, yielding organic acids (such as lactic, acetic, propionic, and butyric acids), branched-chain fatty acids (such as isobutyric, isovaleric, and 2-methylbutyric acids), H_2, CO_2, ammonia, amines, and several other end-products.[18,19] Short-chain fatty acids (SCFA) are, from a nutritional point of view, the major fermentation products. They affect the metabolism, growth, and differentiation of colonocytes, influence the hepatic control of lipids and carbohydrates, and provide the muscles, kidneys, heart, and brain with energy.[18]

The use of probiotics to modulate the activity and composition of the gut microbiota to improve the health status is consolidated. They are defined as "live microbes which when administered in adequate amounts confer a health benefit to the host".[22,23] Even though probiotic microorganisms were acknowledged within different phyla of bacteria and yeasts, the majority of probiotics in use today are bacteria belonging to species of *Lactobacillus* and *Bifidobacterium* naturally colonizing the human colon.[24]

Bifidobacterium is a genus of high G+C (gunosine + cytosine content) Gram-positive bacteria within the phylum of Actinobacteria. Among nearly 50 species recognized so far, the most represented in the gastrointestinal tract of human adults or infants are *Bifidobacterium pseudocatenulatum*, *B. catenulatum*, *B. adolescentis*, *B. longum*, *B. infantis*, *B. breve*, *B. angulatum* and *B. dentium*.[25] Bifidobacteria are abundant gut colonizers and one of the most important health-promoting groups within the colonic microbiota and are largely used as probiotics.[26] They compete with other species of intestinal microbiota and transient organisms for nutrients and attachment sites in the gut. Bifidobacteria are anaerobic saccharolytic fermenters producing lactic and acetic acids, which acidify the large intestine against putrefactive and potentially pathogenic bacteria. Furthermore, they participate in the regulation of intestinal microbial homeostasis, interfere with the ability of pathogens to colonize and infect the mucosa, modulate local and systemic immune responses, stabilize and preserve the gastrointestinal barrier function, produce vitamins, repress procarcinogenic enzymatic activities, and promote the bioconversion of a number of dietary compounds into bioactive healthy molecules.[26−30]

The genus *Lactobacillus* includes almost 200 recognized species of low G+C Gram-positive bacteria within the phylum of Firmicutes.[31] Despite their wide phylogenetic and functional diversity, lactobacilli are invariably anaerobic/microaerophilic, aciduric/acidophilic nonsporulating rods. They are included within the functional group of lactic acid bacteria (LAB), being saccharolytic and strictly gaining energy through the lactic fermentation of carbohydrates. On the basis of the fermentation end-products, they can be classified as obligate homofermentative (giving mainly lactic acid), obligate heterofermentative (giving mainly lactic acid, acetic acid, and CO_2), or facultative heterofermentative.[31]

Lactobacilli occur in a variety of habitats where carbohydrate-based substrates are available. They inhabit plants, plant-derived matrices, and fermented foods (such as dairy products and fermented dough, milk, vegetables, and meats) and are found in the commensal microbiota naturally colonizing diverse niches within the body of humans and animals. In particular, several species are endogenous members of the resident microbiota of the hindgut. Many commensal lactobacilli have been proven to exert a number of beneficial health effects and have attracted considerable attention as candidates for the development of probiotics, although the molecular mechanisms behind these beneficial properties are still under investigation.[32−35] At present, the strains of *Lactobacillus* with the greatest relevance for the manufacture of probiotics and functional foods belong to the species *L. acidophilus*, *L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. reuteri*, and *L. salivarius*.[24,34]

Although at first probiotics were added to foods or consumed as pharmaceutical products to improve the gut microbiota balance, nowadays specific health effects of probiotics have been demonstrated, even though the molecular mechanisms remain largely unknown.[26,34,35] In fact, several studies provided insights into metabolic, trophic, protective, and immune effects of bifidobacteria and lactobacilli, and probiotic strains have been specifically selected to alleviate chronic intestinal inflammatory diseases, to prevent and treat pathogen-induced diarrhea, to manage autoimmune and atopic diseases, to lower cholesterol levels, and to exert antioxidant activity.[36−39] In this context, probiotic strains, selected for the production of specific enzymatic activities, may be exploited to enhance the release of the aglycones, improving the rate of biotransformation toward bioactive metabolites carried out by other intestinal microorganisms.

BIOAVAILABILITY OF NATURALLY OCCURRING CONJUGATED PHYTOCHEMICALS

Many phytochemicals with relevant interest for human health are present in food in the form of esters, glycoconjugates, or polymers, which are not directly bioavailable. The chemical form in which they occur is important because it influences the bioavailability, the biological activity, and, therefore, the physiological effects.[10,40,41] Polyphenols, in particular, are frequently bound to hydrophilic moieties and are generally too polar to be absorbed through passive diffusion by enterocytes in the small intestine.[42] With only a few exceptions, polyphenols bound to sugar moieties cannot be absorbed in their native form and require hydrolysis to their corresponding aglycones.[43]

Isoflavones, which occur abundantly in cotyledons and hypocotyls of soybeans and soy-derived foods, are found as aglycones (daidzein, genistein, and glycitein), glucosides (daidzin, genistin, and glycitin), acetylglucosides (acetyldaidzin, acetylgenistin, and acetylglycitin), and malonylglucosides (malonyldaidzin, malonylgenistin, and malonylglycitin).[5] Due to the lack of active transporters in the intestinal epithelium, both isoflavone glycosides and aglycones are absorbed only via passive diffusion and have poor oral bioavailability.[44] However, isoflavone glycosides are too polar and present lower permeability through the intestinal epithelial membrane, if any, than the corresponding aglycones.[40,45,46]

Likewise, the sugar moiety of glycoconjugated flavonols is a major determinant affecting absorption in the small intestine. Only glucose-conjugated quercetin is rapidly absorbed in the small intestine, probably through a sodium-dependent glucose transporter, whereas the other glycoconjugates (such as rutinose, rhamnose, galactose, arabinose, xylose, and glucuronic acid conjugates) are not.[5,47]

Lignans are involved in plant cell wall formation and ubiquitously occur in many plants. They are found especially in flaxseeds, berries, rye, and a wide range of seeds, fruits, and vegetables.[5,48,49] They can be transformed by colonic microbiota into metabolites that present antioxidative, antiproliferative, antiestrogenic, and antiangiogenic properties. Secoisolariciresinol (SECO), the major dietary lignan, is mostly found as

secoisolariciresinol diglucoside (SDG), which is ester-linked with 3-hydroxy-3-methylglutaric acid and other phenolic compounds (e.g., *p*-coumaric and/or ferulic acid glycosides) in the form of SDG oligomers.[50,51] SDG and the other lignans are not active in the forms as they occur in plants, and their beneficial effects greatly depend on bioconversion into the aglycons and further reactions.[52,53]

Hydroxycinnamic acids (e.g., cinnamic, coumaric, caffeic, and ferulic acids) are one of the major classes of dietary phenolic compounds, occurring in a number of fruits, vegetables, and grains.[5,54] They may be found in foods as free carboxylic acids, esterified with flavonoids, carbohydrates, and quinic and tartaric acid, and, to a minor extent, as amides (with amines or amino acids). Free hydroxycinnamic acids are promptly absorbed through the intestine via both passive and active mechanisms, whereas esters (such as chlorogenic acid) present markedly reduced absorption rates.[54]

GENETIC AND ENZYMATIC CHARACTERS OF PROBIOTIC BACTERIA INVOLVED IN TRANSFORMATIONS OF PHYTOCHEMICALS

Many reactions that transform naturally occurring phytochemicals into bioactive molecules require the activity of different components of the colonic microbiota. Therefore, probiotic lactobacilli or bifidobacteria, if properly selected, can affect the kinetics of transformation of these precursors, thus improving the bioavailability and/or biological activity of natural phytochemicals. From this perspective, information concerning the potential interaction of probiotic bacteria with the dietary compounds is of great interest.

Probiotic strains, and particularly bifidobacteria, bear a number of glycosyl-hydrolases, because they have evolved within the colonic ecosystem, where indigestible oligo- and polysacchardies are the major carbon sources for saccharolytic fermentative bacteria. Thus, they may be involved in the release of aglycones from glycoconjugated forms of polyphenols. Among the diverse glycosyl-hydrolases, β-glucosidases (EC 3.2.1.21) are the most pertinent for the release of the aglycones, because, as formerly described, many phytochemicals are glucoconjugates. In particular, the initial hydrolysis of soy isoflavone glucosides to their respective aglycones is the rate-limiting step in isoflavone absorption, and β-glucosidase activity has been claimed as relevant in relation to isoflavone bioavailability.[45,55−57]

Bifidobacteria are known to produce β-glucosidases. The analysis of *Bifidobacterium* genome and nucleotide sequences for predictable β-glucosidases suggests that *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. pseudocatenulatum* possess two to nine genes encoding β-glu with cytoplasmic or membrane location (http://www.ncbi.nlm.nih.gov/gene; http://www.cazy.org; http://www.cbs.dtu.dk/services/SignalP). The production of β-glucosidases by *B. animalis*, *B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. breve*, and *B. infantis* was correlated to the hydrolysis of glucoconjugate forms of phytochemicals, even though a wide diversity in the activity was found among species and strains and a quantitative relationship with hydrolysis yield could not be established.[58−61] As a matter of fact, cultures of selected members of the genus *Bifidobacterium* are capable of hydrolyzing the glucose moiety from glucoconjugates of isoflavones, flavonols, and lignans.[59,61,62] In particular, β-glucosidase-positive bifidobacteria were active in the hydrolysis of daidzin, genistin, glycitin, kaempferol 3-*O*-glucoside, and SDG into their aglyconic forms.[58,59,61,62]

Lactobacilli are also known to produce β-glucosidase activity.[13,56,63] The analysis of genome and nucleotide sequences of the main probiotic species of *Lactobacillus* revealed that *L. reuteri* and *L. salivarius* lack any sequence annotated as β-glucosidase (http://www.ncbi.nlm.nih.gov/gene; http://www.cazy.org; http://www.cbs.dtu.dk/services/SignalP). Otherwise, a variable number of sequences encoding cytosolic β-glucosidases was found in the genomes of *L. acidophilus* (5−8 sequences), *L. casei* and *L. paracasei* (4−6), *L. plantarum* (9−11), and *L. rhamnosus* (6−8). β-Glucosidase-producing probiotic lactobacilli may contribute to the release of the aglycone from several glucoconjugates phytochemicals, thus improving their bioavailability. Nonetheless, the genus *Lactobacillus* has never been investigated for the hydrolysis of glucoconjugates other than the ones of soy isoflavones.

Up to now, the ability of diverse species of bifidobacteria and lactobacilli to produce β-glucosidase has been mostly applied in food technologies for the production of fermented soy-based foods, enriched in soy isoflavones, as described in several studies.[10,41,58,64−71]

Apart from β-glucosidase, bifidobacteria and lactobacilli are known to produce a number of glycolytic activities that may be potentially involved in phytochemicals activation. However, information on the hydrolysis of conjugated phytochemicals by enzymes other than β-glucosidases is still very scarce and deserves deeper investigation. Rhamnosidases from *L. acidophilus* and *L. plantarum* efficiently hydrolyzed rutinosides and neohesperidosides of flavonols and flavanones such as rutin, hesperidin, and naringin.[72,73] Thus, probiotic strains of *L. acidophilus* and *L. plantarum* may contribute to the release of quercetin, hesperetin, and naringenin (from rutin, hesperidin, and naringin, respectively), which are among the most abundant flavonoids occurring in plant foods. No information is available in the literature about the capacity of *Bifidobacterium* species to hydrolyze the rutinosides rutin and hesperidin. Preliminary results indicate that no bifidobacteria are able to convert rutin into its aglycone quercetin, and only *B. pseudocatenulatum* could transform hesperidin into its aglycone hesperetin to some extent (unpublished data). Members of the genus *Bifidobacterium* were demonstrated to produce enzyme activities that could potentially participate to the metabolism of ginsenosides through the removal of diverse sugar moieties. In particular, α-arabinopyranosidase, β-xylosidase, and β-glucosidase activities, capable of removing diverse arabinose, xylose, and glucose moieties from ginsenosides, were found and characterized in some strains of *Bifidobacterium*, such as *B. breve* K-110 and a strain designated *Bifidobacterium cholerium* K-103.[74−77]

Bacterial species belonging to the genera *Lactobacillus* and *Bifidobacterium* were found to be capable of producing esterase activity, hydrolyzing chlorogenic acid, and releasing caffeic acid, a hydroxycinnamic acid with antioxidant properties, which is much more easily absorbed in the gut.[54,78−81] Lactobacilli capable of performing this transformation were found within the species *L. johnsonii* and *L. gasseri*.[82,83] Among the most common species of probiotic bifidobacteria, the hydrolysis of chlorogenic acid was found only in *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*.[16,83]

SOY ISOFLAVONES AND LIGNANS: TRANSFORMATIONS BY THE GUT MICROBIOTA AND IN VIVO EFFECTS OF PROBIOTICS ON BIOAVAILABILITY

Soy isoflavones and lignans are emblematic examples of phytochemicals that are subjected to bacterial transformations yielding molecules with enhanced biological activity. In fact, colonic bacteria are responsible for the transformation of these molecules into very effective phytoestrogens, which mimic the action of estrogens on target receptors and exert many health benefits against hormone-dependent diseases such as protection against breast and prostate cancers, prevention and treatment of osteoporosis, lowering of hematic cholesterol and lipids, cardiovascular protection, antioxidant activity, and alleviation of menopausal symptoms.[84−86] Specific bacterial groups were demonstrated to be responsible for definite reactions in the biotransformation route of soy isoflavones and lignans toward active phytoestrogens.

One of the major soy isoflavones is daidzein. It is mostly found in the glucoconjugated form daidzin, which is poorly absorbed in the intestine and undergoes extensive transformation in the colon. The first step of daidzin activation is the hydrolysis and the release of the aglycone daidzein, mainly carried out by hydrolytic enzymes from colonic bacteria, such as lactobacilli, bifidobacteria, coliforms, and *Bacteroides*.[15,40,52,87,88] Daidzein can be absorbed through the gut epithelium; otherwise, it can be further transformed by intestinal bacteria into a variety of metabolites with improved or decreased biological activity.[10,11,89,90] In particular, a bacterial reductive pathway could yield *S*-equol, which is the most effective in stimulating an estrogenic response among the isoflavone derivatives (Figure 1). This pathway proceeds with the

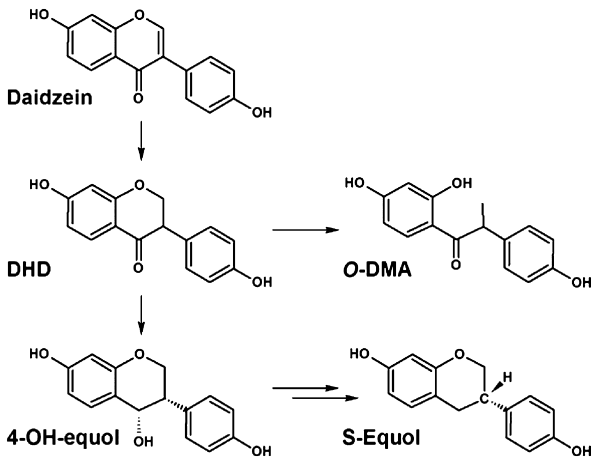

Figure 1. Intestinal bacterial metabolism of the isoflavone daidzein to *O*-desmethylangolensin (*O*-DMA) and *S*-equol.

hydrogenation of daidzein to dihydrodaidzein, carried out by several *Clostridium*-like strains.[11] Dihydrodaidzein constitutes a branch point of two divergent routes of bacterial transformation. One route gives *O*-desmethylangolensin (*O*-DMA), originated by C-ring cleavage, which primes the breakdown of the molecule. The other route proceeds through keto group reduction to 4-hydroxyequol, followed either by reductive rearrangement or by dehydration and subsequent reduction, yielding *S*-equol as the end-product.[89] The role of colonic bacteria in both pathways is well documented, and the composition of the microbiota is responsible for interindividual differences in the capacity to produce *S*-equol and *O*-DMA. In fact, production of *S*-equol occurs only in 30−40% of people, whereas approximately 80−90% can produce *O*-DMA. The species *Eubacterium ramulus*, belonging to *Clostridium* cluster XIVa, can accomplish the C-ring cleavage and is regarded as one of the major isoflavones degrading bacteria in the human gastrointestinal tract.[15] On the other hand, nearly all of the equol-producing bacterial isolates, such as those belonging to the genera *Eggerthella*, *Slackia*, and *Adelcreutzia*, have been classified in the family of Coriobacteriaceae within the high G+C content Gram-positive Actinobacteria.[90−96]

The activation of lignans also depends on the release of the aglycone followed by other bacterial reactions (Figure 2). The

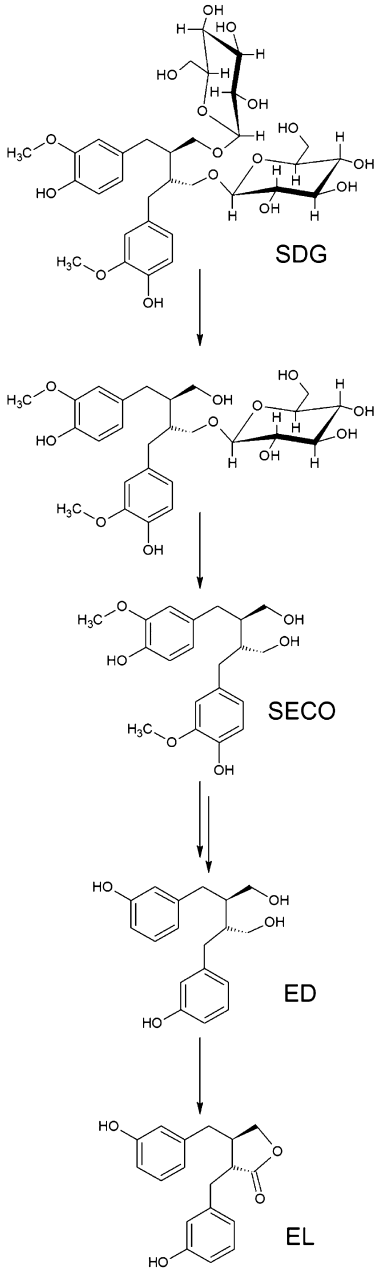

Figure 2. Intestinal bacterial metabolism of secoisolariciresinol diglucoside (SDG) to secoisolariciresinol monoglucoside (SMG), secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL).

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release of SECO from SDG occurs in two steps, with the consecutive removal of the two glucose moieties by bacterial β-glucosidases.[51,62] Bacteriodetes, Clostridiales, and the novel species *Clostridium saccharogumia* are involved in glycoconjugate hydrolysis with release of SECO.[97] Then SECO is further transformed into ED and EL, which exert estrogen-dependent and -independent activity. Production of ED requires demethylation followed by dehydroxylation.[9,98] The species *Peptostreptococcus productus* seems crucial for SECO demethylation, but bacterial isolates capable of this reaction were identified also within other Clostridiales (e.g., *Eubacterium limosum*, *Eubacterium callanderi*, and *Butyribacterium methylotrophicum*); dehydroxylation to ED is a common feature of the species *Eggerthella lenta* within Actinobacteria, but is performed by some Clostridiales as well (e.g., *Clostridium scindens*).[9] The dehydrogenation converting ED into EL is carried out by subdominant species, such as *Lactonifactor longoviformis* in the phylum of Clostridiales, occurring at lower concentrations (in the magnitude order of 10^5 bacteria/g of intestinal content).[9]

For both *S*-equol and ED/EL production, the composition of the gut microbiota is at the basis of interindividual differences in the ability to produce these metabolites.[9,11,15] From this perspective, the impact of probiotic consumption on the transformation of isoflavones and lignans into *S*-equol and ED/EL is of great interest.

The effect of probiotic consumption on the bioavailability of active metabolites of isoflavones and lignans has been investigated. The rationale of these studies always rested on the β-glucosidase activity ascribed to probiotic strains, in agreement with experimental data that consumption of probiotics alters fecal enzymatic activities both in animals and in humans.[99] It has been hypothesized that an increase of β-glucosidase activity, due to probiotic consumption, could improve the aglycone release and increase the flux toward other reactions yielding the bioactive compounds. It is necessary to highlight that probiotic lactobacilli and bifidobacteria seem to be involved only in deconjugation reactions, whereas they do not take part in the reactions transforming the aglycones daidzein and SECO into *S*-equol and ED/EL, respectively.

A few in vivo trials determined whether the consumption of probiotics together with soy-based supplements might improve the bioavailability of active metabolites, enhancing the intestinal absorption and enteroepathic recirculation. In these trials, the subjects were fed soy proteins or soy foodstuffs and treated for 5 weeks with a probiotic yogurt containing 10^8 cfu/100 g daily serving of each of *Lactobacillus* GG, *L. acidophilus*, and *Bifidobacterium bifidus*,[57] for 6 weeks with 3 capsules/day containing 10^9 cfu of *L. acidophilus* DDS+1 and *B. longum* and 15−30 mg of fructooligosaccharide,[100] or for a month with a high load (at least 8×10^{10} cfu/day) of *Lactobacillus* GG.[96] None of these studies established a positive correlation between probiotic consumption and urinary and/or plasma isoflavone metabolite concentrations. In particular, probiotic supplementation did not significantly modify the levels of plasma and urinary isoflavone metabolites, or even negatively affected them.[57,100,101] These results suggest that the transient colonization by different probiotic bacteria can alter the overall microbiota composition and its metabolic activities in a manner that can be hardly predicted and may differ among individuals. Interestingly, in the trial carried out with the highest load of probiotics, levels of isoflavone metabolites were lower than control.[101] Furthermore, although the effect of probiotic consumption did not significantly affect the bioavailability of isoflavone metabolites, in a few subjects their concentration increased or decreased by approximately 9- and 7-fold, respectively, assessing a large interindividual variability in terms of response to the same probiotic formulation.[100]

The effect of probiotic consumption, alone and together with galacto-oligosaccharides, on EL level has been investigated.[102] The probiotic formulation contained a total amount of 2×10^{10} cfu/day of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *B. breve* Bb99 and was associated with a minimum of 120 g/day whole-grain rye bread in addition to the normal diet. Probiotics alone negatively affected the serum EL concentration, whereas the association of probiotics and galacto-oligosaccharides (3.8 g/day) did not determine significant changes. Because this specific combination of strains did not contribute to transformations resulting in EL production, it can be established that the colonization of these probiotic strains likely repressed the growth and metabolic activity of species involved in transformations of lignans. The presence of the prebiotic likely changed the relative amounts of the diverse microbial groups, giving different results than the probiotic alone. Furthermore, these results are consistent with previous evidence that excluded the capability of any of the strains combined into the probiotic preparation to transform the plant lignan 7-hydroxymatairesinol.[103]

DISCUSSION

The food industry continually offers innovative products that satisfy consumer needs and, in some cases, actually persuade the consumer that they have a need. Functional foods containing probiotic microorganisms with scientifically supported health claims for improving the state of well-being and reducing the risk of diseases already constitute a growing market. Because the metabolism of the phytochemicals in the colon is influenced by many factors of the gut environment, exploitation of probiotic bacteria to modify the bioavailability of bioactive compounds can provide new perspectives for nutraceutics.

The in vivo studies herein discussed were based on the assumption that aglycone release could be accelerated by β-glucosidase activity of the probiotic strains. Because the probiotic strains exploited for these trials belong to species that bear genes encoding β-glucosidase, it is conceivable that they produce the enzyme, even though data about activity are not available. However, the presence of several different β-glucosidases in both lactobacilli and bifidobacteria does not ensure the capability of the corresponding enzymes to carry out the hydrolysis reaction of the diverse glycosylated phytochemicals. In particular, steric hindrance can result as a main determinant of substrate reactivity. Enzyme location is probably another aspect that is potentially involved in the difficulties encountered by these probiotic bacteria to hydrolyze the conjugated forms. None of the β-glucosidases annotated in the genome of bifidobacteria and lactobacilli are predicted to be extracellular. Hence, membrane transporters are likely involved in making the substrate available to cytosolic β-glucosidase. Consistently, the inability of certain β-glucosidase-positive bifidobacterial strains to hydrolyze SDG was ascribed to the lack of a transport system enabling SDG to enter the cell and encounter cytosolic enzyme.[62] Similar information is not available for lactobacilli, for which an extensive investigation deserves to be accomplished to conclusively assess their

potential role in aglycone release. In addition, the gut microbiota has intrinsically very high hydrolytic activities,[19,104] and it cannot be excluded that the contribution of the probiotic strains, if any, would be inconsistent.

This overview provides a picture of the potentiality of probiotic bifidobacteria and lactobacilli to take part in the release of aglycones, improving the levels of bioactive metabolites of phytochemicals. However, the outcomes of the few studies carried out administering associations of probiotics and phytochemicals are discouraging. Attention should be given to the evidence that the proposed probiotic strains have not been validated for the major genetic and enzymatic features that can affect phytochemical metabolism. The utilization of probiotic strains, selected for the hydrolysis and activation of specific phytochemicals, needs substantial efforts to be validated.

AUTHOR INFORMATION

Corresponding Author

*(M.R.) Phone: +39 059 2055567. Fax: +39 059 373543. E-mail: maddalena.rossi@unimore.it.

Notes

The authors declare no competing financial interest.

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Isolation of Carotenoid-producing Yeasts from an Alpine Glacier

Alberto Amaretti*[a], Marta Simone[a], Andrea Quartieri[a], Francesca Masino[a], Stefano Raimondi[a], Alan Leonardi[a], and Maddalena Rossi[a]

[a]Department of Life Sciences, University of Modena and Reggio Emilia, via Campi 183, 4100 Modena, MO, Italy
alberto.amaretti@unimore.it

Cold-adapted yeasts are increasingly being isolated from glacial environments, including Arctic, Antarctic, and mountain glaciers. Psychrophilic yeast isolates mostly belong to *Basidiomycota* phylum, such as *Cryptococcus*, *Mrakia*, and *Rhodotorula*, and represent an understudied source of biodiversity for potential biotechnological applications. Since some basidiomycetous yeast genera (e.g. *Rhodotorula*, *Phaffia*, etc.) were demonstrated to produce commercially important carotenoids (e.g. β-carotene, torulene, torularhodin and astaxanthin), the present study aimed to obtain psychrophilic yeast isolates from the surface ice of an Italian glacier to identify new pigment-producers. 23 yeast isolates were obtained. Among them, three isolates giving pigmented colonies were subjected to ITS1/ITS2 sequencing and were attributed to the Basidiomycetous yeasts *Dioszegia* sp., *Rhodotorula mucilaginosa*, and *Rhodotorula laryngis*. The strains were cultured batch-wise in a carbon-rich medium at 15°C until the stationary phase was reached, then the pigments were extracted from freeze-dried biomass using a DMSO:acetone mixture. Visible absorption spectrum and HPLC-DAD analysis revealed the presence of carotenoid pigments. In batch cultures of *Dioszegia* sp., carotenoid production was growth-associated and yielded up to 3.4 mg/L of a molecule exhibiting an *m/z* ratio (568) consistent with the molecular weight of xanthophylls bearing 2 OH groups.

1. Introduction

Cold adapted yeasts, which are capable to survive and thrive at low and/or subzero temperatures, are continuously being isolated from permanently cold ecosystems, including polar and mountain glaciers (Butinar et al., 2007; Rossi et al., 2009; D'Elia et al., 2009; Margesin, 2009). The ecological role of yeasts in glacial environments remains to be deciphered, as to whether they exert an active role in bio-geochemical cycles or they are just preserved in metabolically inactive status. The biodiversity of these organisms is still understudied and underestimated as well, mostly due to the lack of studies using culture-independent approaches aiming to identify non-cultivable strains. Nowadays, most isolates belong to the fungal phylum of *Basidiomycota*, and particularly to the genera *Rhodotorula*, *Cryptococcus*, and *Mrakia*. These yeasts represent an invaluable source of biotechnological resources, in terms of cold active enzymes and unexplored metabolic potential. For example, it has been shown that oleaginous yeasts are very common among psychrophilic isolates and processes for lipid production with cold adapted species of *Rhodotorula* have been described (Amaretti et al., 2010).
A potential application of psychrophilic yeasts is the production of carotenoids. In effect, some basidiomycetous yeast genera (e.g. *Rhodotorula*, *Phaffia*, etc.) were demonstrated to produce commercially important carotenoids (e.g. β-carotene, torulene, torularhodin and astaxanthin) and are referred to as red yeasts (Frengova and Beshkova, 2009). Carotenoids are a family of tetraterpenoids bearing an extensive conjugated polyene system, responsible for absorption of blue light and for their yellow to red color (Fraser et al., 2004). They can be classified as carotens, which are hydrocarbons (such as lycopene, torulene, and α to ε-carotene), or xanthophylls, which bear hydroxyl, keto, and/or carboxyl groups (such as zeaxanthin, lutein, astaxanthin, and torularhodin) (Figure 1). Carotenoids are widespread in nature, being produced as photoprotectants by plants, some photosynthetic bacteria, and some fungi. They are of great interest for food, pharmaceutical, and cosmetic industries, where they find application as vitamin A precursors, pigments, antioxidants, and/or photoprotectants (Fraser and Bramley, 2004).

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Furthermore, they are attracting considerable attention for application in nutraceuticals, and cosmeceuticals, since they exert a number of beneficial effects on health, including maculoprotective, anti-oxidant, anti-inflammatory and anti-tumoral properties (Fraser and Bramley, 2004; Sommer et al., 2012; Rasmussen et al., 2013). In the last decades the increasing demand for natural carotenoids has focused attention on the development of biotechnological processes, alternative to chemical synthetic methods following extraction from plants. Therefore, fermentative processes exploiting the so-called red yeasts are increasingly being described (Nelis et al., 1991; Wang et al. 2007; Frengova and Beshkova, 2009; Moliné 2012).
The present study aimed to obtain new isolates of environmental red yeasts, in order to identify new potential sources for biotechnological production of carotenoids. In this perspective, the yeast biodiversity contained in a surface ice sample from an Alpine glacier was investigated for carotenoid production, because the phylum to which most red yeasts belong, *Basidiomycota*, is particularly frequent among psychrophilic fungi.

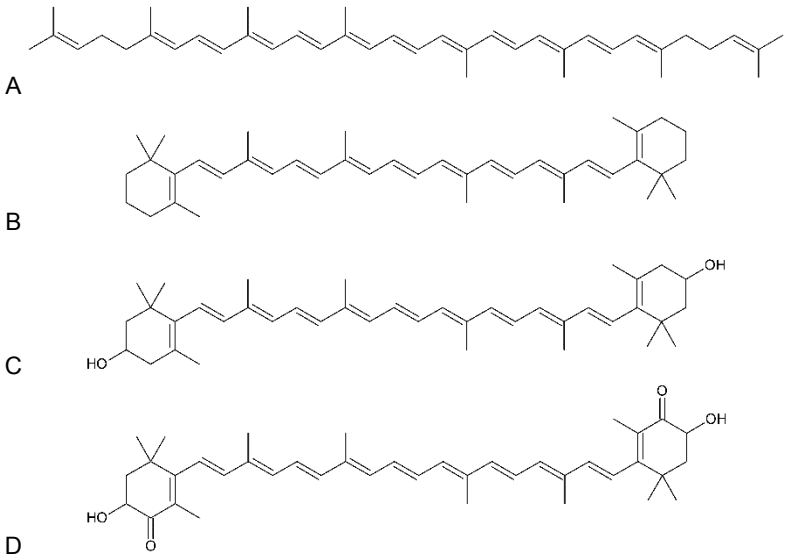

Figure 1: Structure of carotenes (A, B) and xanthophylls (C, D): lycopene (A), β-carotene (B), zeaxanthin (C), astaxanthin (D).

2. Materials and methods

2.1 Yeast isolation and cultivation

Surface ice samples were aseptically collected from an Italian alpine glacier (Tonale Pass, TN, Italy) into sterile containers. Ice samples were allowed to melt at 4°C. 50 mL of melt liquid were centrifuged (5000 rpm, 4°C) to concentrate solids to 5 mL. 0.2 mL of the suspension were plated onto GMY (40 g/L glucose, 3 g/L yeast extract, 8 g/L KH_2PO_4) agar plates supplemented with 50 mg/L chloramphenicol (CAF). Plates were incubated at 5°C until colonies were observed. Colonies with yeast morphology were seeded onto the surface of GMY agar slants and maintained at 4°C. Pigmented yeasts were cultured in shaken flasks of YPD (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and GMY broth at 5 or at 15°C.
Bioreactor batch cultures were performed in a laboratory-scale bioreactor (Labfors, Infors, Bottmingen, Switzerland) containing 2.5 L of GMY medium. The bioreactor was inoculated 10% v/v with a 48-h seed-culture grown in GMY. The culture was kept at 15°C, aerated with 1 v/v/min air, and stirred at 300 to 700 rpm to keep the DOT at 20%. Samples were collected periodically to monitor the growth and to analyze glucose and carotenoids. Biomass dry weight (DW) was determined gravimetrically; glucose was analyzed by HPLC-RID (Amaretti et al. 2010). Biomass/glucose yield coefficient ($Y_{X/S}$) was calculated as the ratio between biomass generated and glucose consumed. The specific growth rate (μ) was calculated by least square regression in the linear tract of the semi-logarithmic growth curve.
All chemicals were purchased from Sigma-Aldrich (Steinnheim, Germany), unless otherwise stated.

2.2 Taxonomic identification

Yeast biomass was collected from 1 mL of grown YPD cultures by centrifugation (10,000 rpm, 5 min, 4°C), was washed twice with water, and was subjected to cell lysis and extraction of genomic DNA using a commercial kit (Master Pure Yeast Dna Purification Kit, Epicentre, Madison, Wisconsin), according to the

manufacturer's protocol. 1 µL of genomic extract was utilized as template for PCR amplification of ITS1 and ITS2 regions within rDNA genes (White et al., 1990). Amplicons were sequenced (Eurofins MWG Operon, Ebersberg, Germany), then taxonomic identification was performed through a comparison with GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed 12.12.2013).

2.3 Pigment extraction and analysis

Biomass was collected from 40 mL samples of GMY cultures (centrifugation at 10,000 rpm, 5 min, 4°C), washed twice with water, and frozen at -80°C. Biomass was lyophilized and extracted under agitation for 16 h at r.t. with a mixture of DMSO and acetone (1:1, v/v). The visible spectrum of the extracts was acquired in the range between 350 and 700 nm. Extracts were analyzed with a HPLC apparatus (1200, Agilent Technology, Santa Clara, CA, USA) equipped with a C18 column and a diode array detector (DAD). Elution was performed at r.t. with 1 mL/min of an isocratic mixture of acetonitrile, THF and water (5:3:2, v/v/v) (Masino et al. 2008). Quantification was performed with HPLC-DAD (λ = 450 nm), using β-carotene as external standard. Information about the molecular weight of carotenoids was obtained with HPLC coupled to a mass spectrometer detector (ESI ion trap).

2.4 Statistical analysis

Experiments were carried out in triplicate. Means were compared with Student's t-test and were considered different for $P < 0.05$.

3. Results and Discussion

3.1 Isolation of psychrophilic pigmented yeasts and growth properties

A sample of surface ice from an Italian alpine glacier was melted and plated onto CAF-supplemented GMY plates. 23 morphologically different yeast colonies arose within 10 d of incubation at 5°C. Three of them, N12, N22, and N23, presented orange to salmon-colored pigmentation and were selected for investigation of carotenoid production. These strains were subjected to amplification and sequencing of ITS1 and ITS2, the hyper variable regions spacing 18S, 5.8S, and 28S rDNA genes. The ITS sequences of N12, N22, and N23 presented high similarity (> 96%) with database ITS sequences from strains of *Dioszegia* sp., *Rhodotorula mucilaginosa*, and *Rhodotorula laryngis*, respectively. Diverse species of *Dioszegia*, and both *R. mucilaginosa*, and *R. laryngis* were already found in polar or mountain glacier-associated habitats (Butinar et al., 2007; Rossi et al., 2009, Connell et al., 2010). *Dioszegia* sp. N12, *R. mucilaginosa* N22, and *R. laryngis* N23 were cultured in aerobic flasks of GMY medium, in order to determine their ability to grow at different temperatures. All the strains behaved as psychrophiles, since they grew abundantly at 5°C and were unable to grow at 30°C. Despite being psychrophiles, the yeasts may be utilized in a biotechnological process, since they were capable of growing at feasible temperatures for technological application (15 and 18°C). All the strains exhibited higher µ ($P < 0.05$) at 15°C compared with 5°C. In *R. mucilaginosa* N22 $Y_{X/S}$ was higher at 15°C than at 5°C ($P < 0.05$), whereas in *Dioszegia* sp. N12 and *R. laryngis* N23, it was similar ($P > 0.05$) at both the temperatures.

Table 1: Growth parameters of Dioszegia sp. N12, R. mucilaginosa N22, and R. laryngis N23 at 5 and 15°C. Values are means (n = 3, SD always < 10%); stars indicate statistically significant difference (P < 0.05).

	$Y_{X/S}$		µ, h^{-1}	
Strain	5°C	15°C	5°C	15°C
Dioszegia sp. N12	0.37	0.37	0.037	0.052*
R. mucilaginosa N22	0.24	0.58*	0.041	0.096*
R. laryngis N23	0.47	0.44	0.024	0.037*

3.2 Pigment extraction and analysis

The pigments of *Dioszegia* sp. N12, *R. mucilaginosa* N22, and *R. laryngis* N23 were successfully extracted with DMSO:acetone from the lyophilized biomass of stationary phase cultures and were subjected to visible spectroscopy in order to obtain preliminary information about the presence of carotenoids. In fact, carotenoids present characteristic visible absorption spectra, with a typical three-peak profile (or two peaks and a shoulder) between 400 and 550 nm, due to their highly conjugated double-bond systems (Young and Hamilton, 1999). The DMSO:acetone extracts obtained from the yeasts yielded visible absorption spectra comparable with that of β-carotene dissolved in the same solvents, but the three-peak pattern was

shifted to higher wavelengths (Figure 2). Thus, it was conceivable that the pigmentation of *Dioszegia* sp. N12, *R. mucilaginosa* N22, and *R. laryngis* N23 was due to the presence of carotenoids.

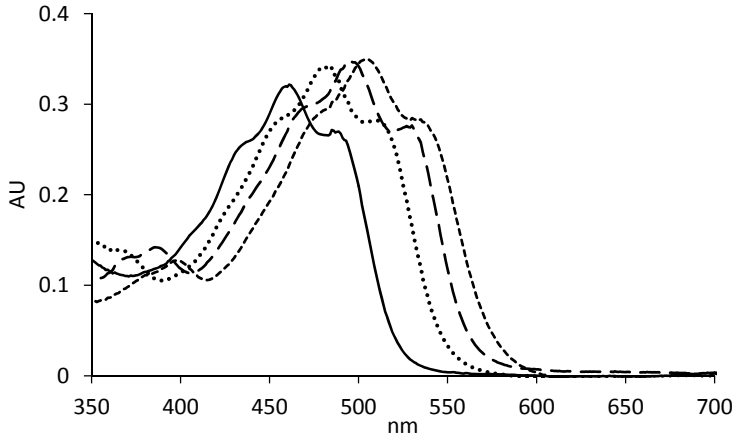

Figure 2: Visible spectrum of β-carotene (solid line) and of the organic extract of Dioszegia sp. N12 (dots), R. mucilaginosa N22 (short dashes), and R. laryngis N23 (long dashes).

The organic extracts of the yeasts were analyzed with reverse-phase HPLC-DAD (Figure 3). *Dioszegia* sp. N12 and *R. mucilaginosa* N22 yielded one major peak each, at 6.4 and 7.6 min respectively, which exhibited a spectrum similar in shape to a carotenoid (λ_{max} = 480 and 500 nm, respectively). *R. laryngis* N23 yielded 3 peaks, at 3.3, 3.6, and 3.7 min, each presenting a spectrum consistent with that of carotenoids (λ_{max} = 490, 480, and 485 nm, respectively). All these peaks were eluted before β-carotene (t_R = 20 min) and were presumably more polar.

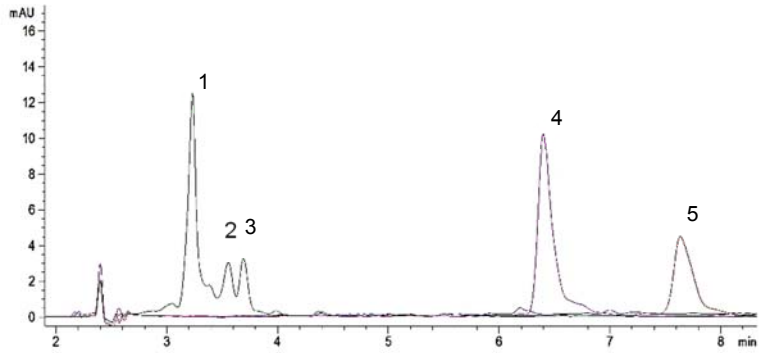

Figure 3: Overlaid HPLC-DAD chromatograms of the extracts from Dioszegia sp. N12 (peak 4), R. mucilaginosa N22 (peak 5), and R. laryngis N23 (peaks 1, 2, and 3). Chromatograms were registered at 450 nm.

3.3 Carotenoid production by *Dioszegia* sp. N12

Dioszegia sp. N12 was selected for deeper investigation of carotenoids production. HPLC-MS analysis revealed that this compound yielded a parent ion with *m/z* = 568, which is consistent with the molecular weight of xanthophylls bearing 2 OH groups (such as lutein and zeaxanthin). Nonetheless, it was excluded that the carotenoid produced by *Dioszegia* sp. N12 was either lutein or zeaxanthyn, since they were eluted with lower retention times. It is remarkable to note that production of xanthophylls other than astaxanthin is not common in red yeasts and has been described only in some species belonging to *Dioszegia* (Madhour et al. 2005). Therefore, the structure of the pigment yielded by *Dioszegia* sp. N12 deserves deeper structural investigation.

Bioreactor batch cultures were carried out at 15°C in order to determine growth and production kinetics (Figure 4). Compared with flask cultures, the strain grew with higher specific growth rate (μ = 0.076 h^{-1}, $P < 0.05$) likely due to improved oxygen transfer. Growth ceased being exponential after 48 h, then continued at lower rate until glucose got exhausted after 160 h. At the end of the fermentation, 17 g/L biomass were

produced, with an $Y_{X/S}$ of 0.43. Carotenoid production was growth associated and occurred in parallel with biomass generation. 3.4 mg/L carotenoid were produced at the end of the fermentation, which corresponded to a content of 0.2 mg carotenoid per g of biomass DW. The amount of carotenoid produced by *Dioszegia* sp. N12 is similar to that obtained in batch cultures with other wild type red fungi (Wang et al., 2007; Madhour et al., 2007; Frengova and Beshkova, 2009). These results make *Dioszegia* sp. N12 a potential candidate for carotenoid production. Thus, possible strain and process improvement deserve further investigating.

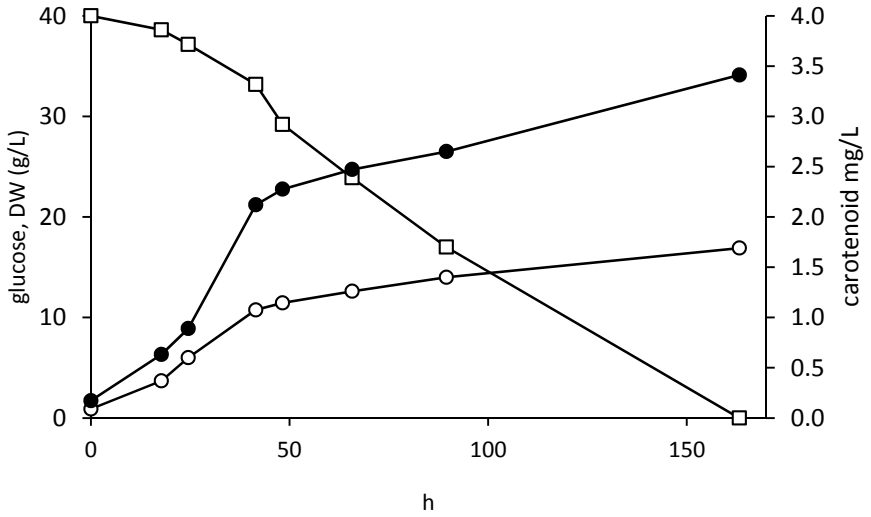

Figure 4: Time-course of glucose (square), biomass (empty circle), and carotenoid (filled circle) during a batch fermentation of Dioszegia sp. N12 in GMY medium. Triplicate experiments were carried out. A representative fermentation is reported.

4. Conclusions

This study confirmed that glacial environment are an underexploited source of biodiversity which deserves being investigated for ecological and biotechnological purposes. One of the new isolates produces a xanthophyll likely bearing two hydroxyl groups, which is quite uncommon among red yeasts. Therefore, this yeast is intriguing for deeper characterization of the carotenoid and for potential strains and/or process improvement.

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