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***Integrated approach to the selection of new
probiotics for human application***

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Chapter 1: General introduction

Probiotics

The definitions given to the term probiotic are numerous. The FAO (Food and Agriculture Organization) and WHO (World Health Organization) define probiotics living organisms which, when administered in adequate amounts, bring benefits to the health of the host. The term probiotic is therefore closely related to the term health: the human intestinal bacterial flora can in fact be easily weakened by different factors such as administration of drugs and stress situations and for this reason it's important to consider a way to support and strengthen the same. Here come into play foods containing probiotics, in line with the definition given above, they are in fact able to optimize the intestinal flora in order to achieve the well-being and a good health.

The International Scientific Association for Probiotics and Prebiotics (ISAPP) organized on 23 October 2013 a meeting of experts on probiotics to revise the definition of the term "probiotic" and to develop clear guidelines for a more conscious use of the term. After the meeting, individual panelists have written a summary document that was approved by all the members before submission called precisely "consensus statements". The panelists established that the FAO/WHO definition of the term "probiotic" remains relevant to validate a new probiotic strain with one modification. A specific strain can be considered as probiotic even if randomized controlled trials are not conducted on it, if the strain belongs to species for which exists a scientific evidence about their beneficial effects on human health. Furthermore, the panel did not agree with the fact of require investigational new drug applications for probiotics food because this activity would increase the costs of research and it is very difficult for products that do not respect pharmaceutical standards. Finally, the panel considers that a strong evidence about the beneficial effect of the probiotic is necessary both at strain-specific or group level and stresses the need to improve communication to the public on the beneficial effects of probiotics.

Our digestive system contains hundreds of viable organisms. There are more than 400 species bacteria that live in the gastrointestinal tract, constituting a real ecosystem. The health of the gastrointestinal flora is not only essential for the correct functioning of the bowel, but it is also important to increment the body's natural defenses against invading bacteria and pathogens.

So food containing probiotics, or friendly bacteria, help to sustain the vitality of our natural defenses.

Human microbiota

The human body is inhabited by a large number of bacteria, viruses and other unicellular eukaryotic organisms. The set of micro-organisms that live in peaceful coexistence with their human host is defined "microbiota" or "normal microflora". The human microbiota consists of a biomass really huge, there are not less than 10^{14} bacterial cells.

The microbiota is an intestinal ecosystem formed by a large number of ecological niches, which are the house of the bacterial population consisting of numerous species and high number of strains. It is closely related with the intestinal mucosa, or with the epithelial interface, that it is, after the respiratory, the largest surface of the body, being approximately 250 - 400 m².

Microbial colonization is a process that begins at birth, when the baby comes into contact with bacteria from the urogenital tract of the mother. Subsequently there is the development of many other microbial species, leading to the establishment of complex interactions between the bacteria themselves and between the latter and the human organism. The microbiota is influenced by many factors such as the composition of the diet of the subject, the body temperature, the use of drugs, the quantity of ingested food and other physiological characteristics; accordingly, it is subject to variations in relation to the changes that occur in the life of an individual.

Man born germ-free. During the first year the intestinal stretch of the baby passes by a condition of sterility to a colonization very dense. Breast milk is an important source of bacteria that influences the development of the intestinal microbiota of the infant. Lactic acid bacteria, bifidobacteria, streptococci and staphylococci are the main groups represented and, between these, the first two are those that will operate positively in different periods of human life. A two-years life of the new born intestinal microflora is almost stabilized.

Any portion of the gastrointestinal tract is colonized by specific bacteria that adapt to local conditions.

The oral cavity due to its characteristics of temperature, pH and nutrient availability is a favorable environment for the growth of microorganisms. The main bacterial genera present in

the oral cavity are *Streptococcus* and *Lactobacillus* and also *Actinomyces* are quite abundant in this cavity; however, there is a considerable variation of the microflora in the case of plate or of oral infections.

In the mouth, in addition to the bacteria, are also commonly present *Archea*, yeasts, especially of the genus *Candida*, and other microorganisms such as mycoplasma and protozoa.

The environment of the stomach has a low pH (less than three), which makes difficult the survival and growth of microorganisms. The gastric flora is quantitatively very poor, so it is possible to define the content of the stomach almost sterile. Only some bacteria are able to multiply in this section of the digestive system; they are acid tolerant and they consist mainly of lactobacilli (*Lactobacillus acidophilus* and *L. plantarum*) and streptococci.

The number of bacterial cells present in the gastrointestinal tract of a mammal shows a continuum increasing, varying from 10^3 bacteria/g in the stomach and duodenum, to 10^4 - 10^7 in jejunum and ileum, to over 10^{12} cells/g in the cecum and in the colon (Figure 1). The acid environment of the stomach has a negative impact on the most bacteria that pass through it, so this is the first defensive barrier to contamination from the outside. Most of the bacteria resides in the lower part of the digestive system, especially in the large intestine, also because in the most proximal tract bile and pancreatic secretions are toxic or not favorable for the growth of the most microorganisms.

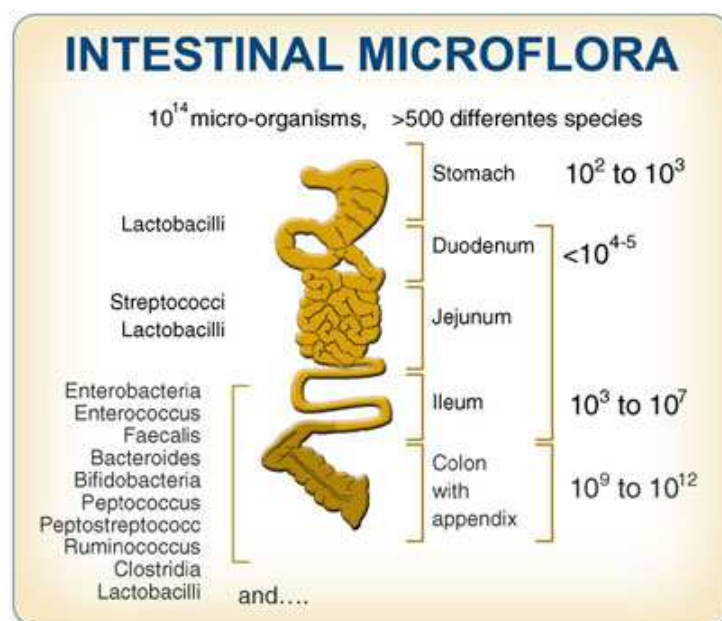


Figure 1: Distribution of intestinal microflora (Customprobiotics.com)

This microflora normally is not pathogenic and helps to maintain the state of health of the host, facilitating the absorption of nutrients, degrading substances potentially harmful or allergenic proteins and generating immune responses such as to prevent inflammation in the intestine (Chin *et al.*, 2000).

It was seen that among the components of the gut flora, lactic acid bacteria, such as bifidobacteria and lactobacilli, are able to exert benefits for the health of the host. Lactobacilli are ubiquitous inhabitants of our body being found in numerous districts: gastric, intestinal, oral, urogenital, etc. They are also an important component of the intestinal microbiota, not so much in quantitatively terms but mainly from the functional point of view, and because they are used in probiotic applications. The intake of lactobacilli as probiotics is aimed to maintain the intestinal microbial flora constantly in balance in order to avoid situations of intestinal dysbiosis, characterized by an alteration of the same with the predominance of pathogenic bacteria.

Lactic acid bacteria

Lactic acid bacteria constitute a large and diverse family of microorganisms that, from the fermentation of sugars, mainly produce lactic acid. These bacteria, essentially ubiquitous, are normally present in food products and are widely used at industrial level because they intervene in many fermentation processes. In particular, they are found in foods and beverages made with plant materials such as sauerkraut, pickles, silage, fodder and beer; they are also agents of the cheese ripening and, together with yeast, are involved in the leavening of bakery products. Some are part of the normal microbiota of the animals and can be ingested by humans as well as probiotics. These microorganisms may have the form of coccus or rod, are Gram positive, catalase negative, unable to move, they don't produce spores, and anaerobic-microaerophilic, in fact they will multiply at low oxygen concentrations. In addition to not possess the catalase they don't even have the enzymes nitrate reductase and cytochrome oxidase; in fact, they don't have a respiratory chain but they have a fermentative metabolism. They are heterotrophic microorganisms, adapted to live on complex substrates and which, as a source of energy, require not only carbohydrates but also vitamins, nucleotides, mineral elements such as manganese and magnesium (used as cofactors of metabolism) and amino

acids. They are in fact very demanding from the nutritional point of view and they require particular regards for nitrogen and vitamin sources while they are being diversified for the carbonaceous sources. Among lactic acid bacteria there are mesophilic and thermophilic species; the optimum pH for growth varies from 7 to 5, otherwise they well tolerate high acidity, some species are in fact able to grow at pH values very low, approximately of three units. This taxa, belonging to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*, includes a variety of genus such as *Abiotrophia*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*.

Among them the genus *Lactococcus*, *Streptococcus* and *Lactobacillus* are particularly important (Makarova and Koonin, 2007).

Genus *Lactobacillus*

Lactobacillus is a genus of Gram positive and rod-shape bacteria belonging to the family of *Lactobacillaceae*. The genus *Lactobacillus* is widely distributed in nature (there are over 100 species) although, like all lactic acid bacteria, it has high nutritional requirements. Lactobacilli can grow in a temperature range between 5°C and 53°C with optimal values of 30 - 40°C. They have an optimum pH for growth of 5.5 to 5.8 but they can still grow even at pH less than 5. These bacteria produce mainly lactic acid by fermentation of sugars but also acetic acid, ethanol, carbon dioxide and other secondary compounds. The production of lactic acid leads to a reduction of the pH of the medium in which they grow, and this acidification of the environment is able to inhibit the growth of certain pathogenic microorganisms. On the basis of how they use glucose during fermentation process they may be divided into homofermentative (if they produce more than 85% of lactic acid from glucose) and heterofermentative, responsible in this case of heterolactic fermentation that products for about 50% lactic acid and for about 50% other substances (they produce lactic acid, carbon dioxide, ethanol and / or acetic acid in equimolar amounts). In relation to the metabolism, the species of *Lactobacillus* can be divided into three groups:

- Obligated homofermentative: include microorganisms that ferment hexoses carbohydrates with production of only lactic acid, they are not able to ferment pentose and they don't produce gas. They are part of this group the species *L. delbrueckii*, *L. acidophilus*, *L. helveticus* and *L. salivarius*.

- Facultative heterofermentative: include lactobacilli which produce lactic acid by fermentation of hexoses but some species, in certain conditions, also produce acetic acid, formic acid and / or ethanol. In addition, they also ferment pentoses with the production of lactic acid and acetic acid. This group includes the species *L. casei*, *L. curvatus*, *L. plantarum*, *L. sakei*.
- Obligated heterofermentative: they ferment hexoses with formation of lactic acid, carbon dioxide, acetic acid and / or ethyl alcohol. They are also able to ferment pentose producing lactic acid and acetic acid. They are included in this group the species *L. fermentum*, *L. brevis*, *L. collenoides*, *L. hilgardii*, *L. fructivorans*.

Lactobacilli are present in different habitats, in addition to the digestive and urogenital human system other habitats are represented by silage, soil, water, cereals and fermented foods (milk, meat and vegetables).

The different species of lactobacilli have adapted differently: they have developed different characteristics associated with the environment in which they live. The broad ecological distribution of lactobacilli makes them an interesting subject of research on genome evolution and lifestyle adaptation. To explore evolutionary mechanisms that determine genomic diversity of different species of lactobacilli, nowadays it is used the comparative analysis of genomes. The analysis of the genome have contributed to a more complete understanding of their phylogenetic position and revealed important details about their specialized adaptation to a specific environment. Van de Guchte *et al.*, 2006, through genome sequencing, have demonstrated the adaptation of *L. bulgaricus* to the milk environment through the protocoooperation with *Streptococcus thermophilus* and these species are in fact the species most used for yogurt production. Also Cremonesi *et al.*, 2013 through the genomic analysis explained the widespread use of *L. helveticus* in cheese technology. About that it has revealed genes responsible for key metabolic functions such as proteolysis, lipolysis, and cell lysis and these genes can facilitate the production of cheese and cheese derivatives. These studies provide insights about mechanisms for genome evolution and lifestyle adaptation of these ecologically flexible and industrially important lactic acid bacteria.

Genus *Bifidobacterium*

Microorganisms of the genus *Bifidobacterium* are non spore-forming, non motile and they can show various shapes, which the most typical ones are slightly bifurcated club-shaped or

spatulated extremities. They are strictly anaerobic, although some species can tolerate low oxygen concentrations and they have a fermentative metabolism. Tissier described these bacteria at the beginning of the twentieth century (Tissier, 1900). They were first included among the family *Lactobacillaceae*, but in 1924 the species *L. bifidum* was reclassified into the new genus *Bifidobacterium*.

The species of the genus *Bifidobacterium* form a coherent phylogenetic group and show over 93% similarity to the 16S rRNA sequences among them (Satokari *et al.*, 2003). This genus is included in the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*, and it includes Gram-positive bacteria with high content of G+C.

All the currently known *Bifidobacterium* isolates are from a very limited number of habitats, that is human and animal GITs, food, insect intestine, and sewage (Felis and Dellaglio, 2007; Ventura *et al.*, 2004). Strains most commonly found in human intestines and feces are those belonging to the species *Bifidobacterium catenulatum*, *B. pseudocatenulatum*, *B. adolescentis*, *B. longum*, *B. breve*, *B. angulatum*, *B. bifidum* and *B. dentium*, and the typical species isolated from functional foods is *B. animalis* subsp. *lactis* (Masco *et al.*, 2005). Often in commercial products are present species other than those typical of the human gut. An example is given by *B. lactis*, a species widely used in commercial products thanks to its greater capacity for survival and resistance to stress.

The genome structure of this group of microorganisms remains largely unexplored; Milani *et al.*, 2014 sequenced the genomes of 42 subspecies belonging to the genus *Bifidobacterium* and they used this information to explore the genetic picture of this bacterial group. They suggest that the evolution of this genus was substantially influenced by genetic adaptations to obtain access to glycans, indicating in this way that this mechanism represents a strong evolutionary force in shaping bifidobacterial genomes.

Another application of the analysis of the genome revealed the genetic elements responsible for the use of carbohydrates by bifidobacteria of the intestinal microbiota. Particularly, Milani *et al.*, 2015 have performed the analysis of the genome of 47 strains of bifidobacteria showing that, in this bacterial genus, the genes for the catabolism and degradation of carbohydrates are better represented compared to those found in other bacteria of the intestinal microbiota and this ability to degrade glycans reflects the availability of carbon sources of the human gut.

“Non-conventional” probiotics

In recent years new species of bacteria with probiotic properties have been discovered. Among them there is the species *Faecalibacterium prausnitzii*, an important commensal bacterium of the human gut flora. In healthy adults, the species *F. prausnitzii* represents more than 5% of the bacteria in the intestine, making it one of the most common gut bacteria. In the last few years an increasing number of studies have described the importance of this commensal bacterium as a component of the healthy human microbiota demonstrating that lower than usual levels of *F. prausnitzii* in the human gut have been associated to dysbiosis in several human disorders (Miquel *et al.*, 2013). Particularly, it was shown that a decrease in the levels of this bacterial species is closely related to Crohn's Disease as demonstrated by Wright *et al.*, 2015. They studied the taxonomic shifts in patients with this inflammatory bowel disease revealing significant changes in microbial composition: the abundance of *Bacteroidetes* is increased and *Firmicutes* decreased in Crohn's disease compared with healthy controls. *Enterobacteriaceae*, specifically *Escherichia coli*, is enriched while *F. prausnitzii* is found at lower abundance.

In order to develop a novel probiotic, several strains of this bacterial species were isolated and characterized by Foditsch *et al.*, 2014 showing that the concentrations of the short chain fatty acids acetate, butyrate, propionate and isobutyrate in the culture media change in the presence of bacterial growth. They observed a significative reduction in the concentration of acetate followed by a concomitant increase in the concentration of butyrate, suggesting that the isolates are able to consume acetate present in the media and producing butyrate. This is a positive effect because butyrate has many benefits to the colonic epithelial cells and so the selection of strains that produce higher amounts of butyrate is extremely important for the development of a new potential probiotic.

Another “non-conventional” probiotic is represented by the species *Akkermansia muciniphila*, a species of the new genus, *Akkermansia*, proposed in 2004 by Derrien *et al.* *A. muciniphila* is a Gram-negative, strictly anaerobic, non-motile, non-spore-forming and oval-shaped. It is able to use mucin as its sole source of carbon and nitrogen, and able to colonize the gastrointestinal tracts of human. Extensive research is being undertaken to understand its association particularly with inflammation (Everard *et al.*, 2013). Infact, *A. muciniphila* is believed to have anti-inflammatory effects in humans, and studies have shown inverse relationships between *A. muciniphila* colonization and inflammatory conditions such as appendicitis or irritable bowel

syndrome. In one study, reduced levels of *A. muciniphila* are correlated with increased severity of appendicitis. In a separate study, patients with irritable bowel syndrome were found to have lower levels of *A. muciniphila* in their intestinal tract than individuals without this syndrome (van Passel *et al.*, 2011).

Probiotics: guidelines for use in foods and food supplements

In order to protect the health of consumers, foods containing probiotics are subject of attention, as confirmed by the elaboration of international guidelines aimed to safeguard the quality of the products.

Ministerial guidelines (revision of 2013) define first the requirements required to use microorganisms as probiotics. They must in fact be sure to use in humans and to be alive and vital in such quantity as to allow multiplication and their activity in the intestine (for reasons that are explained after).

Amount of microorganisms

Ministerial guidelines specify that the minimum quantity of microorganisms able to temporarily colonize the intestine is 10^9 living cells for strain and for day. The recommended daily dose of the product must therefore possess a charge in viable cells equal to 10^9 for at least one of the strains present in the product. This is a topic of much debate because despite regulatory orientation is the one just mentioned, some leaders of the sector say that in a mixed product all species must have a charge of at least 10^9 .

Safety of probiotics

To ensure the safety of the microorganism used, it's necessary an identification of the same on the strain level. First of all, for safety and efficacy reasons it is important that each strain under study is clearly identified, in fact different bacterial strains belonging to the same species may play different beneficial actions in the host. The species can be identified by determination and analysis of the DNA sequence coding for the 16S rRNA or through the hybridization of nucleic acids, the strain can be characterized by PFGE (Pulse Field Gel Electrophoresis). Some of these methods are technically obsolete, however, they are still required from regulatory point of

view. Some of these techniques are no longer used because they are time-consuming and not cost efficient but they are highly reliable because they allow to obtain highly reproducible profiles unlike other new methods.

The clear identification of strains and their amount in the products are nowadays subject to careful regulation given the frequent cases of misidentification and mislabeling that occurring. An example is shown by the control made by Aureli *et al.*, 2010 who tested 72 of probiotic food supplements produced and distributed on the Italian market during 2005-2006. They have shown that 87% of the products analyzed do not comply with the guidelines and the differences were both quantitative and qualitative (number, types and viability of microorganisms). Moreover, even though most labelled supplements indicate the presence of *Bifidobacterium bifidum*, this organism was detected only rarely and always as dead cells. This study is a clear demonstration that on the Italian market, there are commercial probiotic products that do not correspond to what is written on the label and consequently to what the Italian guidelines suggest.

In order to consider safe a bacterial strain is necessary a reliable taxonomical identification as described above, and the evaluation of the sensitivity to antibiotics. As is known, in fact, the phenomenon of bacterial resistance to antibiotics is often based on the presence in the bacterial cells of mobile genetic elements, such as plasmids and transposons, which can be transmitted from one organism to another promoting the horizontal spread of resistance. Particularly the intrinsic resistance to antibiotics is not a problem, the problem arises when the resistance determinants may be transferred to other bacterial strains; evaluation of antibiotic sensitivity is necessary to ensure the absence of acquired or potentially transmissible resistances. For the evaluation of the bacterial safety EFSA (European Food Safety Authority) has introduced the concept of QPS ("Qualified Presumption of Safety"), in practice it was developed a list of bacterial groups suitable for QPS assessment: for them it is not necessary safety assessment but only the determination of sensitivity to antibiotics (EFSA, 2013).

It is on the basis of this regulation and the international community on probiotics that researchers should be directed to develop products really useful to the consumer, taking into account that different types of consumers need different probiotics: probiotics are in fact

influenced by various factors such as age of the host, his physiological / pathological conditions and his diet. It is also necessary to increase the effectiveness of products containing probiotics and, for that, it is important to pay attention in each phase of the production: from the selection of bacterial strains to the classification and identification of the same and then proceed to the safety assessment and the determination of the functional characteristics of the strains.

Selection and characterization of *Lactobacillus* and *Bifidobacterium* strains: “conventional screening”

A pool of bacterial strains is initially characterized from the taxonomical point of view and for safety for use in human (“conventional screening”) and then only bacterial strains that possess these features of safety were subjected to a more specific screening, depending on the application to which they will be destined (targeted screening application-focused).

They will therefore be intended to a process of "selection funnel" aimed at the realization of a series of *in vitro* tests to assess their applicability as probiotics for the promotion of human health.

The most common bacteria used as probiotics belong to the genus *Lactobacillus* and *Bifidobacterium*. The selection process of a strain to be used as a probiotic is based on a multi-step approach:

According to FAO/WHO guidelines it is necessary to identify the microorganism to species/strain level because probiotic effects are strain specific (FAO/WHO, 2006). Further characterization of strains is important especially for safety assessment, in fact in order to guarantee fermented products and food supplements that are safe for consumption, some characteristics of the novel lactobacilli and bifidobacteria strains must be studied to ensure their safety.

Within the “Conventional screening” several *in vitro* tests are conducted for a further characterization of strains to ensure that the bacteria are able to reach the intestine alive and vital, an important requirement to ensure their effectiveness. In Figure 1 are represented the main phases of the “conventional screening”.

Figure 1. Guidelines for the Evaluation of Probiotics for Food Use.

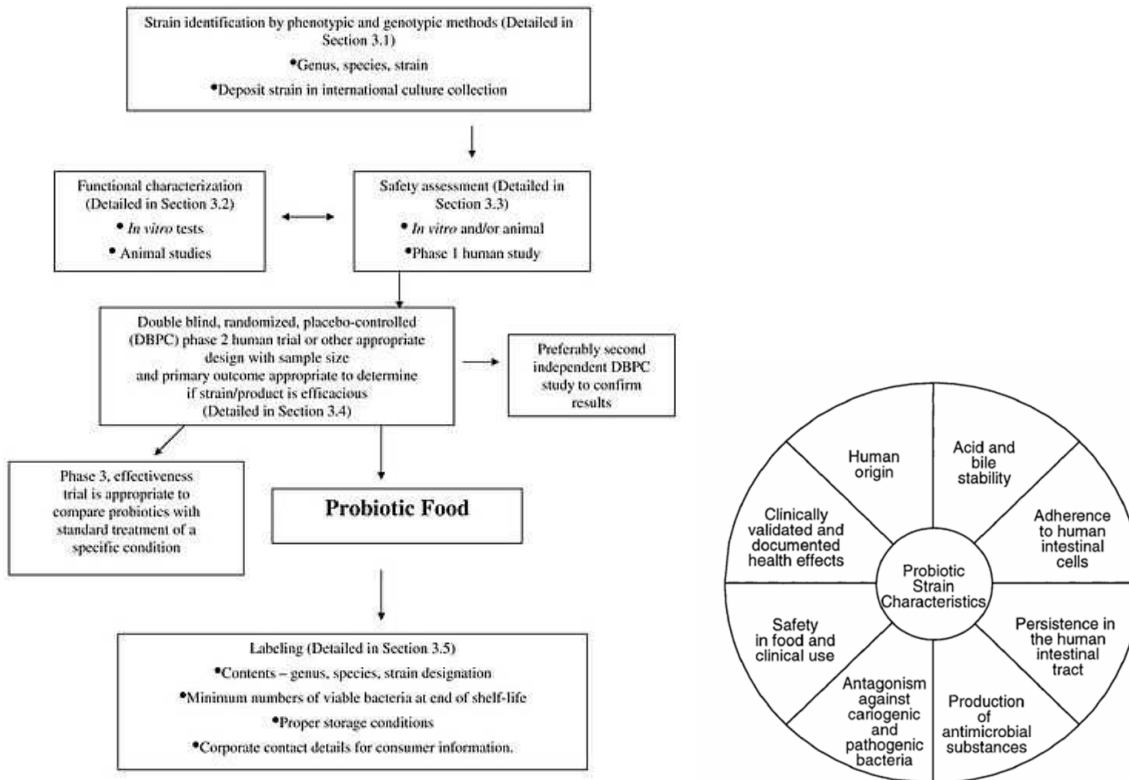


Figure 1: Main phases for identification and characterization of probiotics (Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food London, Ontario, Canada, 30 April – 1 May 2002)

It is well accepted that an effective human probiotic should be of human origin and this is due to the fact that human intestine is different from those of animals, so strains isolated from animal intestine may not be suitable for humans (O’Sullivan, 2001).

For the isolation of new strains, classical cultivation techniques must be employed, particularly selective media and specific culture conditions are used for the isolation of strains from human fecal samples (Delgado *et al.*, 2006).

Identification and typing

In the last decade molecular methods, mainly based on the analysis of nucleic acids by using polymerase chain reaction (PCR) amplification, have been developed for identifying probiotics (Ben-Amor *et al.*, 2007).

The study of ribosomal RNA genes is the most common method for the identification of bifidobacteria and lactobacilli. Bacterial ribosomes are composed by proteins and three

ribonucleic acids: 5S RNA, 16S RNA, and 23S RNA. The analysis of the 16S rDNA allows to discriminate all *Bifidobacterium* and *Lactobacillus* species and their respective subspecies and biotypes (Germond *et al.*, 2002; Matsuki *et al.*, 2003; Ventura *et al.*, 2006) by sequencing fragments of DNA previously amplified by “universal” or group-specific primers. In addition to provide a precise identification, comparison of the 16S rRNA gene sequences allows to understand the evolutionary relationships among the distinct species. The analysis of 16S rDNA sequences has shown that the classification of lactobacilli in three metabolic groups (obliged homofermentative, facultative heterofermentative, obliged heterofermentative) is not in accordance with their evolutionary relationships. In fact *Lactobacillus* species can be grouped into several groups and don't form a coherent phylogenetic unit (Felis and Dellaglio, 2007; Satokari *et al.*, 2003).

Methods based on the PCR are widely used and allow the differentiation between strains of the same species, by examining patterns generated by amplification of DNA fragments these methods offer a potential for probiotic strain typing. Particularly, REP (repetitive extragenic palindromic) PCR examine specific patterns of repetitive DNA elements. REP sequences are short DNA fragments detected in the extragenic space, and they are dispersed in the bacterial genomes (Ventura *et al.*, 2003; Tobes and Ramos, 2005).

Recently, more robust typing methods have been applied to species and strains of *Lactobacillus* and *Bifidobacterium* such as the multilocus sequence typing (MLST) scheme. This method made use of an automated DNA sequencing procedure to characterize the alleles present at different housekeeping gene loci. As it is based on nucleotide sequences, it is highly discriminatory and it provides unambiguous results. This powerful technique has been applied for phylogenetic studies of strains of *Lactobacillus* and a variant of MLST, called multilocus variable-number tandem repeats analysis (MLVA) was used for the subtyping of *L. casei* / *L. paracasei*.

Also the genome sequence analysis is nowadays used as a new taxonomic technical approach and it provides insights on bacterial evolution thus influencing bacterial taxonomy. Genomic sequence information has in fact been proposed for defining a new genomic-phylogenetic species concept for prokaryotes. For example, comparative genomics has supported the idea that the lactobacilli don't form a coherent phylogenetic group but it seems that some species (*L. salivarius*, *L. plantarum*) are more closely related to *Enterococcus faecalis* than to other lactobacilli. An example of genomic techniques is the comparative genome hybridization (CGH)

that can be used to determine the genome content of a bacterial strain for which is not known the genome sequence.

Antibiotic sensitivity

Lactic acid bacteria are naturally resistant to many antibiotics, but in most cases the resistance is not transferable by horizontal gene transfer (HGT). However when the antibiotic resistance is plasmid-associated it can be transferred to other species and genera, so it is important to select strains lacking the potential to transfer genetic determinants of antibiotic resistance. About that, Mathur and Singh observed that LAB used in probiotics may be a source antibiotic-resistant genes which could be potentially transferred to pathogenic bacteria (Mathur and Singh, 2005). Therefore they suggested systematic screening for antibiotic resistance in probiotic strains: In the case of presence of antibiotic resistance the approach to follow is suggested by Courvalin, 2006, consisting in the identification of the resistance gene, evaluation of the ability to transfer the resistance, characterization of the biochemical mechanism of resistance and elucidation of the genetic basis for resistance. If the resistance feature is not associated with a mobile genetic element, the risk of transfer of resistance would be assessed as low.

The experimental methods used to test the sensitivity to antibiotics are described in Chapter 2.

Survival to GIT stressing conditions

The ability to survive to stressful gastrointestinal tract (GIT) conditions (low pH and high bile salts concentrations) is an important criteria used for the selection of probiotics strains. The transit of probiotics present in food through the GIT takes variable times and is submitted to different stressful conditions. After mastication, the first barrier that bacteria must overcome is the low pH of the stomach with values ranging from 1 to 3. Into the duodenum the pH value rises to 6-6.5 but bile salts reach concentrations ranging from 1.5 to 2% during the first hour of the digestion (Noriega *et al.*, 2004). For the screening of probiotics so it's important to simulate *in vitro* these GIT conditions: a low pH value and a high bile concentration are tested for variable times in order to determinate the survival of the strains under test.

Targeted screening focused on the application

“Conventional” screening described above consists of *in vitro* experiments that are mainly important to assess the safety of probiotic strains. However, it is realized that these tests, including identification of strains, safety assessment and characterization of some activities (ability to grow in conditions of low pH and in the presence of bile acids) are not accurate to predict the potential use and the functionality of probiotic strains. So, it’s necessary to expand the knowledge of specific properties of tested strains.

Knowledge about intestinal microbiota, nutrition, immunity, and genetics in health and disease has increased in the past years. This information helps to develop new probiotic strains with disease-specific functions. Probiotics are not a uniform group of microorganisms with health benefits: the efficacy and the properties of each specific strain should be assessed individually rather than as a group of probiotics mainly because different strains exert different effects.

Then, “conventional” screening must necessarily be followed by a screening depending on the application based on the development of platforms for functional characterization, adopting screening criteria for assess if the potential new probiotic is suitable for use in certain areas of consumer health (oncological diseases, cardio-vascular, metabolic, etc.).

Until few years ago, there was the idea that the same strain could be used for different applications, this is not plausible in fact a strain with specific features is usually employed for a specific application. Nowadays in fact is widely supported the thesis that, for the same strain, you can not have an effective use for different applications but, for each application, you must choose the most suitable strain.

This targeted screening is closely related to the benefits of probiotics on human health.

An increasing number of studies have shown that probiotic bacteria are in fact able to positively influence the state of health, thanks to the numerous activities that they carry out, in particular: maintaining a balance in the intestinal microflora, protection against intestinal pathogens and modulation immune response leading to an improvement in allergies food and autoimmune disorders (Figure 2).

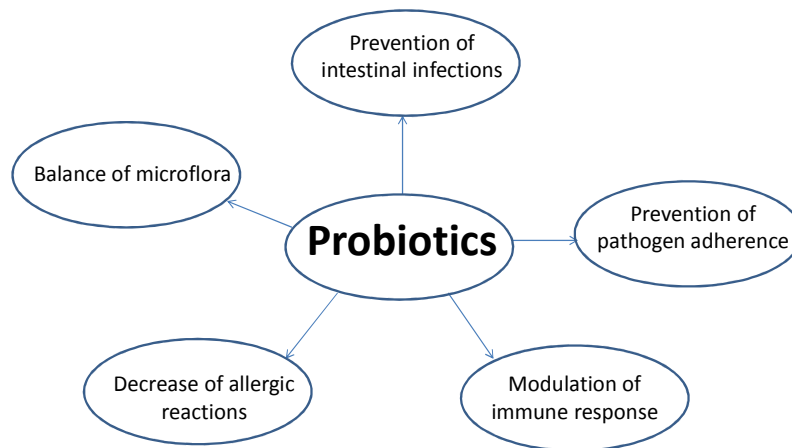


Figure 2: Mechanisms of action of probiotics

Prevention of pathogen adherence

The adhesion of pathogens to the intestinal mucosa appears to be crucial for the beginning of the infection process.

Pathogenic bacteria are in fact able to adhere to the intestinal epithelium, colonize with detrimental effects to epithelial cells.

The microbiota present in the intestine provides a barrier to the adherence of pathogenic bacteria and various mechanisms have been proposed to explain this defensive action of probiotics. Some authors have suggested that the production of organic acids, such as acid lactic and acetic acid by probiotics, especially by some lactobacilli strains, lowers the intestinal pH and inhibits the growth of pathogens. Moreover, the same authors have seen that probiotics stimulate peristalsis that, indirectly, removes pathogens, accelerating the speed of transit in the intestine.

Another mechanism supported by various studies is the ability of probiotics to compete with pathogens in adhering to the intestinal mucosa (Arquès *et al.*, 2015). Some probiotics can inhibit the adhesion of pathogens to their binding sites on the surface of the intestinal membrane by competing for binding.

A study of Gopal *et al.*, 2001, has confirmed that some lactobacilli, like *L. rhamnosus* DR20, *L. acidophilus* HN017 and *B. lactis* DR10, are able to adhere to intestinal cell lines *in vitro* and to decrease the colonization and the entry of pathogens into the intestinal mucosa.

Prevention of diarrhea and intestinal infections

It's well known that various strains of lactobacilli can be used to prevent or treat acute diarrhea caused by *Escherichia coli*, *Salmonella* or *Shigella* and this effect is mainly due to the production of bacteriocins by different strains of probiotics used. A study of Davoodabadi *et al.*, 2015 has shown the ability of strains of *L. fermentum*, *L. plantarum* and *L. paracasei* to inhibit the growth of enteropathogenic bacteria including strains of *Shigella flexneri*, *Shigella sonnei*, *Salmonella enteritidis* and *Yersinia enterocolitica*.

Some authors have used *L. rhamnosus* GG added to yogurt, to treat diarrhea associated with the treatment by antibiotics as demonstrated by a recent study that evaluated the efficacy of *L. rhamnosus* GG in the prevention of antibiotic-associated diarrhea both in children and in adults (Szajewska and Kołodziej, 2015). The administration of antibiotics can lead to an imbalance in the microbiota and a net reduction of the intestinal "beneficial" microflora, the main responsible of the resistance to the colonization of pathogens. The results of this study showed that humans volunteers that received probiotics with the antibiotic showed lower diarrhea compared to individuals that consumed only pasteurized yogurt as control (Siitonen *et al.*, 1990).

Efficacy against diarrheal disease is well documented also with other probiotics strains, such as *L. reuteri*, *L. casei* and *Saccharomyces boulardii* (Huang *et al.*, 2002; Van Niel *et al.*, 2002). Probiotics are widely used for prevention and treatment of diarrhea more in children than in adults (Guarino *et al.*, 2015): the administration of probiotics, such as *B. bifidum*, *Streptococcus thermophilus* and *L. rhamnosus*, has proved very useful also as prophylaxis to prevent nosocomial diarrhea in children (Szajewska *et al.*, 2001).

The incidence of Crohn's disease and ulcerative colitis collectively called syndrome IBD (inflammatory bowel disease), is constantly increase in industrialized countries. The changes in lifestyle, including a better hygiene and a reduction in the consumption of foods containing bacterial enzymes, may alter the correct microbial balance necessary for the development of a correct intestinal immune system (Shanahan, 2004). This can lead to immune reactions towards the intestinal bacterial flora, and this could be the first step leading to the development of IBD and other disorders related to wrong immune response. Several experimental evidence in fact suggest that both Crohn's disease and ulcerative colitis are caused by hyperactivation of the immune system against intestinal flora, leading to a chronic inflammatory state and a consequent mucosal damage (Shanahan, 2002).

The balance between pathogenic and beneficial bacteria in the intestine represents a protection against abnormal immune responses that lead to inflammation.

For this reason a good therapeutic strategy against IBD is a modification of the microflora in favor of probiotics (Fedorak and Madsen, 2004).

Experimental studies carried out *ex vivo* on intestinal mucosa of patients suffering from Crohn's disease have shown that certain *Lactobacillus* strains, including *L. casei*, can induce a decrease of the release of TNF- α (anti-inflammatory signal) by the inflamed mucosa.

Moreover experiments conducted on rats which spontaneously develop colitis, have shown that oral administration of *L. reuteri* or *L. plantarum* 299v did significantly decrease the symptoms of the disease (Schultz *et al.*, 2002). Also Torres-Maravilla *et al.*, 2015 demonstrate the potential of a strain of *L. sanfranciscensis* to treat IBD. Often the symptoms of IBD were treated by using a mixture of probiotics as demonstrated by a study of Yoon *et al.*, 2015 that evaluated the effect of a probiotic mixture composed by *L. acidophilus*, *L. rhamnosus*, *B. breve*, *B. lactis*, *B. longum*, and *S. thermophilus* on the changes in fecal microbiota and IBD symptoms. They demonstrated that after 4 weeks of administration of this multi-species probiotic mixture the fecal concentration of most probiotic strains increased and the diarrhea-symptoms in IBD patients improved.

Overall, these results show that probiotics act by modulating immune response in IBD thanks to regulatory cytokines, and this suggest an important role for these bacteria for the treatment of IBD.

Stimulation of the immune system and beneficial effects on allergic reactions

Among the various beneficial effects exerted by probiotics very important is their capacity of interaction with the immune system (Jespersen *et al.*, 2015). For this reason it is essential that the immune system recognizes the parts of the microbiota as self and express a tolerance towards them, this tolerance is also possible thanks to the fact that the bacteria of the microbiota do not express virulence factors (Aureli *et al.*, 2011).

The thesis that probiotics may influence immunity thus exerting a beneficial role in the treatment of human diseases is nowadays a topic of great interest.

There are in fact extensive evidence that suggest that lactic acid bacteria are able to stimulate both innate and acquired immune response, the lymphocyte function and the production of antibodies and cytokines (Gill *et al.*, 2000; Pessi *et al.*, 2000).

First of all, the ability of probiotics to modulate lymphocyte populations has been demonstrated by some authors who observed an increase in the population of T-helper lymphocytes in rats treated with *L. casei* (Perdigon *et al.*, 1999).

Furthermore it was seen that some strains of probiotics are able to increase the number of populations of neutrophils and macrophages, as well as to stimulate the activity of the cells natural killer (Gill *et al.*, 2000; Matsuzaki and Chin 2000) which are the first line of defense thanks to their cytotoxic activity exercising against antigens (Ley *et al.*, 2008). Moreover, some strains of lactobacilli induce dendritic cells (DC) maturation that pass through epithelial cells and capture antigens from the lumen.

About the effect of probiotics on the production of antibodies, numerous studies have shown that the treatment with some strains of probiotics is able of enhancing the immune response antigen-specific against natural infections and immunizations, in particular probiotics act by increasing the production at mucosal level of immunoglobulin A (IgA), the first line of defense against pathogenic bacteria and viruses that daily are inhaled and ingested. A more recent study investigated the IgA increase induced by a strain of *L. plantarum* and demonstrated that this strain increased the IgA level of Peyer's patch (PP) cells, although its mechanism of action is not clear yet. They demonstrated that taking concentrations of 0.03% or 0.3% of *L. plantarum* powder for 4 weeks, caused an increase of IgA in the small intestine of the mice (Kikuchi *et al.*, 2015). Despite these results indicate the ability of this strain to maintain mucosal immunity, it is necessary to better understand its mechanism of action in order to use this strain in functional food. Another study of Sakai *et al.*, 2014 demonstrated that a strain of *L. gasseri* stimulates dendritic cells to promote the production of TGF- β , IL-6, and IL-10, all critical for IgA production from B cells activating in this way the signal to produce IgA in the mouse small intestine.

For many years the production of cytokines was associated only with the response against infections and it has been given little attention to the fact that lactic acid bacteria could induce the production of cytokines even in conditions of perfect health. The effect most studied on the immunomodulatory activity of probiotics regards precisely the expression of cytokines, both pro- and anti-inflammatory, at both intestinal and systemic level. Several *in vitro* studies show an increase in pro-inflammatory cytokines such as IL-12 and tumour necrosis factor-alpha (TNF- α) in the presence of probiotics as evidenced by a study of Nishibayashi *et al.*, 2015 where it

was investigated the role of three LAB strains in inducing the production of interleukin-12 from human monocytic cells. Particularly the study was conducted by using a strain of *E. faecalis*, a strain of *L. gasseri* and one of *B. breve* and it was demonstrated that a treatment with RNase A of heat-killed LAB significantly decreased the IL-12 production of human cells demonstrating that the single stranded RNA (ssRNA) of LAB is a strong inducer of IL-12 production from human monocytes. Other authors confirmed the trend of lactobacilli in increasing the production of inflammatory cytokines, finding an increase of IFN- γ and IL-12 in human peripheral blood mononuclear cells treated with *L. johnsonii* and *L. sakei*, while the level of IL-10 is not seemed to increase (Haller *et al.*, 2000).

It was also reported an activity of probiotics in inducing the expression of anti-inflammatory cytokines. About that, an *in vivo* study of Wang *et al.*, 2015 on patients in dialysis evaluated the effect of probiotics on inflammatory markers such as interleukin IL-6 and TNF- α that are elevated in patients with this disease and the impact of the same bacteria on the anti-inflammatory cytokine IL-10. They found that a treatment with a strain of *B. bifidum*, *B. catenulatum*, *B. longum* and *L. plantarum* caused a decrease in the levels of serum of pro-inflammatory cytokines TNF- α , IL-5 and IL-6 while levels of serum of IL-10 significantly increased.

These studies confirm the role of the probiotics in the modulation of gene expression associated with the immune system and inflammation. Specific strains of Bifidobacteria and Lactobacilli influence the gene expression of mucins, nuclear factor and interleukins leading to an anti-inflammatory response in the presence of enterocytes in culture (Plaza-Diaz *et al.*, 2014). Moreover, probiotics interact with the surface of antigen-presenting cells *in vitro* causing the downregulation of pro-inflammatory genes that are linked to inflammatory signaling pathways, while other anti-inflammatory genes are upregulated. These effects of probiotics are widely studied in animal models while information about the impact of probiotics on gene expression in human intestinal cells are very scarce. There is the need of further clinical studies to elucidate the mechanism of action of probiotics both in healthy humans and in patients with chronic diseases. These types of clinical studies are necessary for addressing the influence of these microorganisms in gene expression associated with the immune response to finally better understand the role of probiotics in the prevention and treatment of disease.

Then accumulating evidence suggests that probiotic bacteria play an important role in modulating various aspects of immunity and the ability to regulate this type of responses is very useful from the clinical point of view, for example for immunoprophylaxis or, more generally, to increase immunity against pathogens of different nature.

There are several studies that demonstrate a role of probiotics also in autoimmune and allergic disorders.

Food allergies are chronic disorders of growing importance in countries more developed, and they are mainly due to an uncontrolled immune response against specific antigenic determinants present in the environment or in the food.

Considering the anti-inflammatory and immunomodulatory effects exerted by probiotics, it was suggested the use of probiotics as a new strategy for the control of inflammation and allergic reactions, because they are able to favorably alter the microflora of the host and modulate the intestinal immune response.

It is well known that an adequate colonization of the intestine by the bacterial flora in the first years of life is responsible of the proper balance between the Th1 cells and Th2 ones, which provides protection against allergies (Kalliomaki and Isolauri, 2003; Bischoff and Crowe, 2004). The intestinal microflora can potentially promote anti-allergenic processes, it can in fact stimulates the production of TGF- β and IL-10, resulting in promotion of the oral tolerance (Christensen *et al.*, 2002; McGuirk and Mills, 2002). It was in fact observed a decrease in the inflammatory immune response to food antigens in allergic individuals following treatment with probiotics (Cosenza *et al.*, 2015).

Gut-brain axis

The term probiotic is always associated with the intestinal environment and with the maintenance of its eubiosis. However in recent years has arisen the idea that probiotics can also affect brain functions by helping to improve or prevent disorders such as depression and anxiety. The ability of the microbiota to communicate with the brain is demonstrated by the fact that a decrease in beneficial flora leads to a deterioration of the gastrointestinal and neuroendocrine relationships causing diseases. The collaboration between the brain and the gastrointestinal tract is fundamental for maintaining homeostasis and it is regulated at the level of central and enteric nervous systems (Cryan and O'Mahony, 2011). Perturbation of these

systems has as a consequence an alteration in the stress-response and behavior (Rhee *et al.*, 2009). So the enteric microbiome has an impact both on the gut that on the brain and for this reason it was coined the phrase “gut-brain axis”.

It is certain that bacteria in the human gut have the ability to produce molecules with neuroactive functions which could affect the brain. Some typical bacteria of the human GIT are in fact able to produce many neurotransmitters and neuromodulators and, among them, *Lactobacillus* spp. and *Bifidobacterium* spp. have been reported to produce γ -aminobutyric acid (GABA) and, especially bacteria of the genus *Lactobacillus*, have been reported to produce acetylcholine and histamine. These secreted neurotransmitters from bacteria in the intestinal lumen may induce epithelial cells to release molecules that modulate neural signal in the enteric nervous system and consequently the functioning of the brain and the behavior of the host (Wall *et al.*, 2014). It was demonstrated that different strains of *Lactobacillus* and *Bifidobacterium* are able to produce GABA when growing in the presence of monosodium glutamate. GABA is a neurotransmitter that in the brain is involved in states of anxiety and depression through the regulation of different physiological processes (Schousboe and Waagepetersen, 2007). Another study of Messaoudi *et al.*, 2011 assessed the effect of the combination of *L. helveticus* and *B. longum* on rats and demonstrated that these probiotics reduced anxiety in animals. Although the mechanism of action is not known it has been shown that different probiotic strains are able to modulate the level of inflammatory cytokines (Brenner and Chey, 2009) and to decrease oxidative stress thereby reducing depression and anxiety. A bacterial species able of modulating the state of depression by inhibiting the production of pro-inflammatory cytokines is the *B. infantis* (Desbonnet *et al.*, 2008).

Several studies have also shown that the use of a combination of probiotics obtained by combining different bacterial genera or different species of the same genera may be more effective than a mono-species supplements (Chapman *et al.*, 2011). However, some strains can compete with others in the execution of their functions so the use of a mixture of strains to obtain a greater effect requires careful verification in the preparation of the mixture itself.

Moreover, these evaluations were usually carried out using animal models, in particular were used germ-free mice or rats. These animals do not have any bacterial contamination, and thus

offer the opportunity to study the effect of the complete absence of the intestinal microbiota on behavior. Although these animals are useful for research in the field of neuro-gastroenterology, the results obtained from animal studies can not be extended to human individuals since they are not representative of the real situation of the human population. Probiotics studies in human are still scarce but available data look promising. For example a study of Benton *et al.*, 2007 found that a treatment of three weeks with a drink based on milk containing *L. casei* Shirota improved mood scores in individuals with symptoms of depression.

So these data suggest an ability of probiotic strains to modulate some aspects related to brain and behavior, but these results obtained in animal models have to be confirmed in human and there is also the need to understand the molecular and cellular basis of this gut-brain communication mediated by microbiota.

Market of probiotics

The worldwide interest in the field of probiotics is very high, thanks to the many new products launched every year on the market, the interest of researchers in demonstrating the real property and consumers, more and more attentive to health products. The growing awareness of consumers and their attention to new concepts of health and well-being in recent years, favored a significant growth of the global market for probiotics, thereby increasing the level of interest and investment of industries, researchers and producers. Over 500 new probiotics products were introduced in the last decade, although, not everyone receives the same level of success in the market.

Estimates of Euromonitor International at the end of 2014 confirm the positive trend of the markets for vitamins and food supplements in the main countries of Western Europe, with the only exception represented by Germany. For all the other markets, the medium annual growth rate varies from 2.7% in Italy to 3.9% in the UK. Regarding the expenditure per capita, the highest level is reached in Italy with 29,50 €. Italian consumer is followed by the Belgian one with 27,20 €. In addition, in Italy the trend of the category is driven by probiotics and minerals. Concluding given the rapid evolution of the probiotic market in recent years, companies operating in this field have the main objectives of identify new applications for their products and differentiate the end use to try to further expand and to support the market.

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Aim of the thesis

In recent years, the European market for probiotics has evolved rapidly, thanks mainly to the more interest that consumers give to those food that are marketed primarily for their health benefits ("functional" products). Although there are many potential benefits of some probiotics on human health, there are few bacterial strains for which there is a scientific evidence that demonstrates the beneficial effect of the same. For this reason it is necessary an intensive research aimed at discovering new bacterial strains and to investigate the mechanisms of action and their health properties.

During these three years I have done an higher education and research apprenticeship, a contract for the acquisition of the title of PhD, which includes both practical training in a company that a secondary university education. This type of contract introduced with the Legislative Decree n. 167/2011 is addressed at young people under the age of 30 years, lasts three years and allows young people to enter into the job market with regular employment, gaining professional experience and achieving, at the same time, the title of PhD. This contract has allowed AAT to personalize my research plan taking into account working specific needs and skills requirements and especially considering the needs of the market.

AAT has for years been engaged in screening new potential probiotic strains owning a large collection of bacteria; the objective of my doctoral research is to contribute to the evolution of screening criteria by combining conventional approach to newly designed platforms especially targeted towards health-promoting applications. The specific objective of the work was to isolate one or more bacterial strains able to survive in the acidic environment of the stomach, able to survive and multiply in the intestine even in the presence of bile salts and able to produce during fermentation a high bacterial mass, with a good capacity of resistance to the treatments of freezing and lyophilisation and a significant stability in powdered form.

Bacterial strains found to possess these features were then subjected to a more specific screening, depending on the application to which they will be destined. They will therefore be intended to a process of "selection funnel" aimed at the realization of a series of *in vitro* tests to assess their applicability as probiotics for the promotion of human health.

The isolation of a bacterial strain with these features represents an important goal because products that contain this organism could be used not only in adults but also in those persons

belonging to the age-group typically characterized by a compromised immune system; the intestinal flora of children and elderly is often subject to attack by pathogenic bacteria, for this reason there is the need to ingest a food enriched with the most suitable bacterial strain (which may be different depending on the subject) in order to contribute to the normal function of the immune system and to restore the situation of eubiosis.

Chapter 2 – Selection of probiotic candidates following conventional in-vitro criteria

Selection of probiotic candidates following conventional in-vitro criteria

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Introduction

Among the microorganisms involved in the preparation of fermented foods, lactic acid bacteria are widely used in the production of a great variety of fermented foods, such as yogurt (Adolfsson *et al.*, 2004), meat, vegetables, bakery products, silage and other, contributing in various ways in determining their characteristics and their stability.

The increased use of LAB in food production as probiotics has raised safety issues: one of them is the nature of acquiring and distribution of antimicrobial resistance (Ammor *et al.*, 2007). Overuse of antibiotics have contributed to the emergence of resistant bacteria. Antibiotic resistance is the phenomenon by which a bacterium is resistant to the activity of an antimicrobial drug. Over the years this phenomenon has become increasingly important, especially for those bacteria whose sensitivity to certain drugs seemed undisputed. This phenomenon has already been studied extensively in foodborne pathogenic bacteria (Rahimi *et al.*, 2010; Gousia *et al.*, 2011), but in recent years also fermented products have received particular attention as potential vehicles of the spread of antibiotic resistance determinants, which could be transferred to pathogenic bacteria by horizontal via (Perreten *et al.*, 1997; Franz *et al.*, 1999; Klein *et al.*, 2000). In particular, this role of diffusion of the antibiotic resistance, which consequently favors the selection of resistant bacterial species, is carried out by commensal bacteria (Snary *et al.*, 2004) commonly present in food. Recent work had focused on strains of *L. paracasei*, one of the species mainly present in fermented products that, despite its probiotic properties (Andriulli *et al.*, 2008; Chouraqui *et al.*, 2008), appears to play a pathogenic role (Cannon *et al.*, 2005). Determinants responsible of antibiotic resistance are in fact been identified in this species (Huys *et al.*, 2008; Kyriacou *et al.*, 2008; Devirgiliis *et al.*, 2008; Zonenschain *et al.*, 2009), which can act as a vehicle for the spread of these genes to pathogenic bacteria (Teuber and Perreten, 2000).

As *L. paracasei*, other commensal bacteria can exchange genes for antibiotic resistance (Teuber *et al.*, 1999; Salyers *et al.*, 2004; Ammor *et al.*, 2007); this explains the importance of systematically test the strains for the presence of these genes before using them as probiotics or as starter cultures in food.

In 1998 the participants involved in The Lactic Acid Bacteria Industrial Platform (LABIP) workshop on probiotics supported criteria for the selection and assessment of probiotic lactic acid bacteria (Guarner and Schaafsma, 1998), two of them are the evaluation of resistance to

gastric acidity and bile toxicity. Then, bacterial strains considered safe for use in humans, were evaluated also for these parameters under laboratory conditions. Particularly resistance to gastric acidity is important because probiotic bacteria, before reaching the intestinal tract, must first survive through the stomach (Henriksson *et al.*, 1999). The survival of the bacteria in the presence of gastric juice depends on their ability to tolerate low pH. Several LAB isolated from sausages, e.g., *L. sakei*, *L. plantarum*, *L. pentosus*, can tolerate such acidic conditions (Erkkilä and Petäjä, 2000; Klingberg *et al.*, 2006; Pennacchia *et al.*, 2004). Therefore, some authors propose that strains intended for probiotic purposes should be screened for tolerance to pH 2.0 in a culture medium acidified (Klingberg *et al.*, 2006).

For using lactic acid bacteria as effective probiotics it's also necessary to evaluate their ability to resist the effects of bile acid (Lee and Salminen, 1995), compounds that are synthesized in the liver from cholesterol and are secreted into the duodenum in the conjugated form (Hoffman *et al.*, 1983). These acids then undergo chemical modifications (for example deconjugation) in the colon as a result of microbial activity (Hill and Draser, 1968). It has been shown that both forms (conjugated and deconjugated bile acids) exhibit antibacterial activity inhibiting the growth of pathogenic bacteria such as *Escherichia coli*, *Klebsiella* and *Enterococcus* (Stewart *et al.*, 1986). However, Gram-positive bacteria are found to be more sensitive than Gram-negative bacteria (Floch *et al.*, 1972).

However microorganisms can reduce the emulsifying effect of the bile salts by hydrolyzing them with bile salt hydrolase enzymes (Erkkilä and Petäjä, 2000). This enzymatic activity has been described in some lactobacilli isolated from intestinal environment such as *L. acidophilus*, *L. casei*, and *L. plantarum* (Gilliland and Speck, 1977). Moreover in recent years, other LAB strains such as *L. sakei*, *L. pentosus*, have been shown to resist bile salts (0.3% (w/v) oxgall) (Klingberg *et al.*, 2006).

The aim of this work was to study the occurrence of antimicrobial resistance among newly isolated strains of *Lactobacillus* and *Bifidobacterium* from healthy subjects. Some other strains considered in the course of this PhD thesis had already been previously isolated and characterized for their susceptibility to antibiotics and the relative data were confirmed during the thesis. New probiotic candidates evaluated as safe for the use in humans have been subjected to further *in vitro* tests aimed to evaluate their probiotic properties. Given the fact that probiotic strains are intended for human consumption, the most relevant determinations

are the ability of the strains to grow in the presence of gastric juice and bile salts. Finally we detect the genetic identity of the new strains isolated by an amplification of repeating elements of genome DNA.

Materials and methods

Isolation of LAB

One hundred and fifty strains of LAB were isolated from fecal samples or vaginal swabs. All adults and infants were healthy by the moment of the study, without a history of gastrointestinal or vaginal infections. Feces were collected with a sterile spatula into a container and transported to the laboratory. Serial dilutions were prepared from the material. Aliquots of 0,1 ml from 10^7 and 10^9 dilutions of the studied material were inoculated in Petri dishes with MRS (De Man, Rogosa and Sharpe) agar medium (BD Difco, USA), complex medium suitable for the growth and isolation of bacteria belonging to the genus *Lactobacillus*. For the isolation of bacteria belonging to the genus *Bifidobacterium* was added, to the MRS medium, L-cysteine hydrochloride (Sigma-Aldrich, Germany) at a concentration of 0,5 g/l. The dishes with cultures were incubated in jar using Anaerocult A (Merck, Germany) for the production of an anaerobic environment. It is in fact composed by kieselguhr, iron powder, citron acid and sodium carbonate and, when it is put into the jar, the chemical mixture chemically binds oxygen, creating an oxygen-free environment and a CO₂ atmosphere.

The purification step was performed using the technique of streak on the same culture media. Primary screening of *Lactobacillus* and *Bifidobacterium* strains was carried out by evaluating the morphology of the colonies. All types of colonies suspected to belong to *Lactobacillus* or *Bifidobacterium* genus were isolated in order to obtain pure cultures: first in solid nutrient medium, then in liquid nutrient medium for subsequent preparation of frozen bacterial cultures.

DNA extraction

For use in Polymerase Chain Reaction (PCR) DNA from bacteria was extracted using Whatman™ FTA™ CloneSaver™ Cards (GE Healthcare Life Sciences, UK). CloneSaver Cards are designed for the long-term, room temperature storage of DNA from bacterial cultures, resuspended colonies or glycerol stocks. DNA stored on CloneSaver Cards is ready for PCR amplification. The area is

formatted in a 96-well configuration and its pink color changes to white upon application of samples. 5 µl of an overnight bacterial culture ($OD_{600}=1,8$) were pipetted into the center of one of the 96 printed circles; when the sample is applied, the pink color of the spot turned white. Samples were let air-dry for one hour prior to process, then a 2,0 mm punch was removed from the target sample using a Harris Micro-PunchTM and transferred to a microcentrifuge tube suitable for PCR. The punch was washed by adding three times 200 µl of sterile FTA Purification Reagent (GE Healthcare, UK) and by adding twice 200 µl of TE buffer (10 mM Tris, 0,1 mM EDTA, pH 8,0) to the tube. All traces of TE buffer were removed and the punch is left to dry. PCR amplification is made directly from the punch.

Identification of isolated strains

The putative LAB isolated were further identified by PCR-based 16S rDNA sequencing using a pair of universal primers P0 (5'-GAGAGTTTGATCCTGGCTCAG-3') (Invitrogen, USA) and P6 (5'-CTACGGCTACCTTGTACGA-3') (Invitrogen, USA) (Di Cello and Fani, 1996).

The PCR products were separated by electrophoresis on a 1,0% (wt/vol) agarose gel (Segetic, Germany) in 1x Tris/acetate/EDTA (TAE) buffer. The gel was visualised after staining with ethidium bromide (Sigma-Aldrich, Germany) under ultraviolet light. PCR products were sequenced at BMR Genomics and then, comparing the sequences obtained with the sequences present in the database (BLAST RDP.), it has been possible to characterize the bacteria at the species level.

Typing by rep-PCR

To ensure that lactobacilli isolates are different from each other and different from other strains already present on the market or in bacterial collections, it was made a rep-PCR (repetitive extragenic palindromic-PCR) amplification using the primer (GTG)₅ (5'-GTG GTG GTG GTG GTG-3') (Invitrogen, USA) or, alternatively, the pair of primers REP1R-Dt (5'-IIINCGNCGNCATCNGGC-3') (Invitrogen, USA) and rep2-Dt (5'-NCGNCTTATCNGGCCTAC-3') (Invitrogen, USA), as proposed by Versalovic *et al.*, 1994. In order to discriminate bacteria that belong to the genus *Bifidobacterium*, rep-PCR was conducted using the primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Invitrogen, USA) as proposed by Masco *et al.*, 2003. These primers were tested for their ability to discriminate between strains of LAB and for their reproducibility.

Polymerase chain reaction was conducted using the MegaMix (Microzone, UK) that contains Taq polymerase (recombinant) in 1,1x reaction buffer (2,75 mM MgCl₂) with 220 µM dNTPs and stabiliser. The total volume of the reaction is 25 µl: the desired volume of the MegaMix is added into the PCR tube, they have been added also two opposing oligonucleotide primers 50 µM and 1 µl of extracted DNA. In particular, a mixture containing all components of the reaction except DNA is made and aliquoted to individual tubes. At room temperature, the template DNA is added in each tube and PCR amplification is performed in an automated SimpliAmp thermal cycler (Life Technologies, USA) with an initial denaturation step (95°C, 7 min) followed by 35 cycles of denaturation (90°C, 30 s), annealing (40°C, 1 min) and extension (65°C, 8 min), and a single final extension step (65°C, 16 min). Polymerase chain reaction conducted using primer BOXA1R differs from the cycle described above and it was conducted with the following parameters: 7 min initial denaturation at 95°C, 30 cycles of 94°C for 1 min, 52°C for 1 min and 65°C for 8 min, followed by a single final extension step (65°C, 16 min). For the screening of DNA fingerprint patterns, 15 µl of each PCR reaction is electrophoresed at a 80 V constant voltage in 2,2% agarose gel in 1x TAE and then DNA was visualised under ultraviolet light, followed by digital capturing of the image using a Kodak Gel Logic 200 imaging system (Kodak, USA).

Evaluation of resistance to gastric juice

New isolated bacterial strains were subjected to an *in vitro* test aimed to assess their ability to resist to the gastric juice. The test was conducted by counting on agar medium at different times of contact the liquid culture of the strain previously placed in contact with a solution of pepsin (Sigma-Aldrich, Germany) at a concentration of 3 g/l and pH 2,0 (Charteris *et al.*, 1998). As a positive control it was used *L. rhamnosus* GG.

Particularly, cultures of bacterial strains were grown overnight in 10 ml of MRS broth (BD Difco, USA) (with L-cysteine hydrochloride 0,5 g/l if the bacterium belongs to the genus *Bifidobacterium*) and subcultured by using 1% (vol/vol) of the inoculum. The cultures were subsequently centrifuged at 7,000 g for 15 min, washed once in an equal volume of 0.25x ringer's solution (Sigma-Aldrich, Germany), and subsequently centrifuged 7,000 g for 15 min as proposed by Corcoran *et al.*, 2005. 0,2 ml of washed cell suspension were admixed with 1,0 ml of simulated gastric juice and incubated at 37°C for 90 min. Samples were taken at 1, 90 and 180 min, serially diluted in maximum-recovery diluent (MRD) (BD Difco, USA), plated on MRS

medium or MRS medium added of L-cysteine hydrochloride 0,5 g/l, and incubated at 37°C in anaerobic conditions for 72 h to determine the viable cells that are therefore able to survive in an acid environment such as the stomach.

Evaluation of resistance to bile salts

The bile tolerance of the respective isolates was examined by slightly modifying the method proposed by Gilliland *et al.*, 1984. The liquid culture of each strain was used to inoculate (1% v/v) 10 ml of MRS broth medium (with L-cysteine hydrochloride 0,5 g/l if the bacterium belongs to the genus *Bifidobacterium*) and, in parallel, the same volume of the medium containing bile salts (Sigma-Aldrich, Germany) at a concentration of 0.3%. The growth of the strain in the two culture media was estimated by recording absorbance at 600 nm, because optical density (OD) is a parameter directly proportional to the growth of the microorganism. In particular, the OD was measured at zero time, after 16 hours of incubation at 37°C and after 24 hours of incubation at the same temperature. In the case of bifidobacteria absorbance was recorded also at 48 and 72 hours of growth. *L. rhamnosus* GG, strain resistant to bile salts, was used as control.

Antibiotic susceptibility evaluation

The antimicrobial resistance of a strain to each antibiotic testing was determined as the MIC, defined as the minimum concentration of antibiotic that is required to inhibit the growth of a microorganism. The MIC was determined through the use of the method of microdilution, according to the indications of the method ISO 10932: 2010 IDF 223: 2010 "*Milk and milk products -Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB)*". The bacterial sensitivity was tested for 9 antibiotics to which the European Food Safety Authority (EFSA) has indicated breakpoints (*Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary Importance*. EFSA Journal. 2012; 10(6):2740), reference values to assess the sensitivity / resistance of a microorganism to a given molecule having an antibiotic action.

The document ISO 10932 (version 2010) indicates the scale of concentrations to be used for each antibiotic. It is based on a series of dilutions obtained by doubling each time the concentration and this concentration depends on the antibiotic in question. The stock solutions of antibiotics are diluted in sterile water and, for having in the microplate the same final

concentrations reported in the document ISO 10932, it is necessary to prepare dilute solutions at a double concentration; these solutions are in fact further diluted 1:1 (obtaining the desired final concentration) when the microplate is inoculated with the bacterial cultures. Bacterial inocula are prepared streaking on appropriate agar medium strains to be tested and diluting some of the colonies grown in liquid medium in order to reach the optical density value indicated in the above mentioned document. Finally, microplate was prepared. It has 96 wells arranged in 8 rows and 12 columns. All wells of the first column are inoculated with the medium inoculated with the strain to be tested (the medium inoculated with the strain represents the positive control). While all wells of the last column are filled with only the medium (the culture medium sterile without inoculum represents the negative control), the remaining wells are filled with the antibiotic to be tested at the concentration 2× previously prepared and inoculated with the bacterial strain under study. The microplate is arranged so that each row presents only one antibiotic, from its lower concentration to the higher one. The document ISO 10932 (version 2010) indicates also strains whose MICs values are known, so for this test I used as reference strains *L. paracasei* ATCC 334 and the *B. longum* DSM 20219.

Results

Identification of isolated strains

A total of one hundred and fifty strains were isolated from healthy adults and infants. Each strain was identified by partial sequencing of 16S rDNA. Among lactobacilli from fecal samples were isolated especially *L. plantarum*, *L. rhamnosus*, *L. paracasei* and *L. fermentum* while in vaginal swabs the species that predominate are *L. crispatus*, *L. jensenii*, *L. mucosae*, *L. vaginalis*, *L. acidophilus* and *L. gasseri*. Regarding bifidobacteria, the most incidence were *B. bifidum*, *B. longum* and *B. animalis lactis* while *B. adolescentis*, *B. thermoacidophilum*, *B. breve* and *B. pseudocatenuatum* were more rare. Species identification of isolated strains showed that, in some cases from the same patient, can be isolated two or more strains of the same species.

To ensure that the strains isolate are different from each other and different from other strains already present on the market or in bacterial collections, it was made a rep-PCR, a rapid method for strain differentiation. When rep-PCR profiles of the studied bacteria had 100% identity by electrophoretic band number and weights, these strains were considered the same

bacterial strain, and *vice versa*, when rep-PCR produced products with different profiles of the bands, the isolated strains were considered as different strains that belong to the same *Lactobacillus* or *Bifidobacterium* species.

The fig. 1, 2, 3, 4 show the rep-PCR profiles of different strains of *L. paracasei* (Fig. 1), *L. plantarum*, *L. fermentum* (Fig. 2), *L. mucosae*, *L. rhamnosus* (Fig. 3) and *L. gasseri* (Fig. 4).

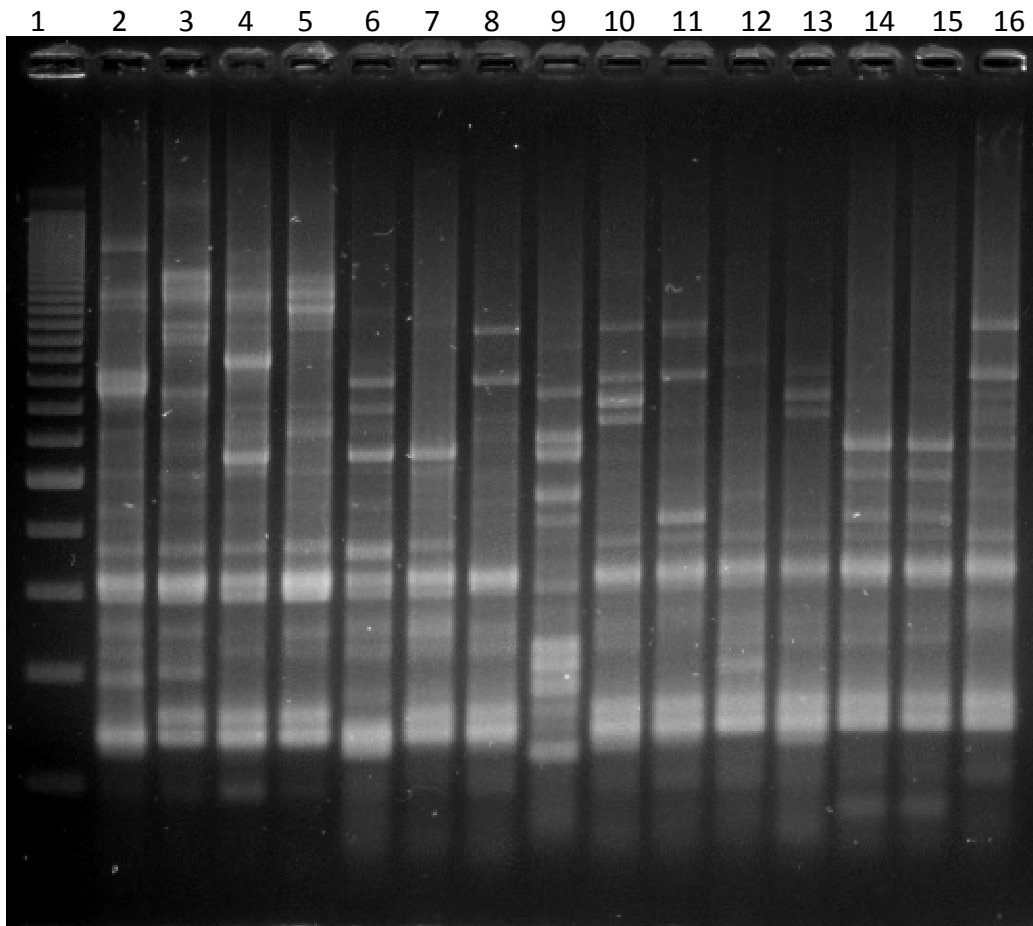


Fig. 1: rep-PCR profiles of *L. paracasei* strains obtained with primer (GTG) (in bold strains isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 ***L. paracasei* LMG S-27487**
- 3 ***L. paracasei* CBA-L101**
- 4 ***L. paracasei* CBA-L144**
- 5 ***L. paracasei* CBA-L145**
- 6 *L. paracasei* ATCC 27216
- 7 *L. paracasei* ATCC 334
- 8 *L. paracasei* B21060
- 9 *L. paracasei* TH0034
- 10 *L. paracasei* DN114001
- 11 *L. paracasei* F19
- 12 *L. paracasei* ST11
- 13 *L. paracasei* shirota
- 14 *L. paracasei* CLV1
- 15 *L. paracasei* CV1
- 16 *L. paracasei* LAFTIL26

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

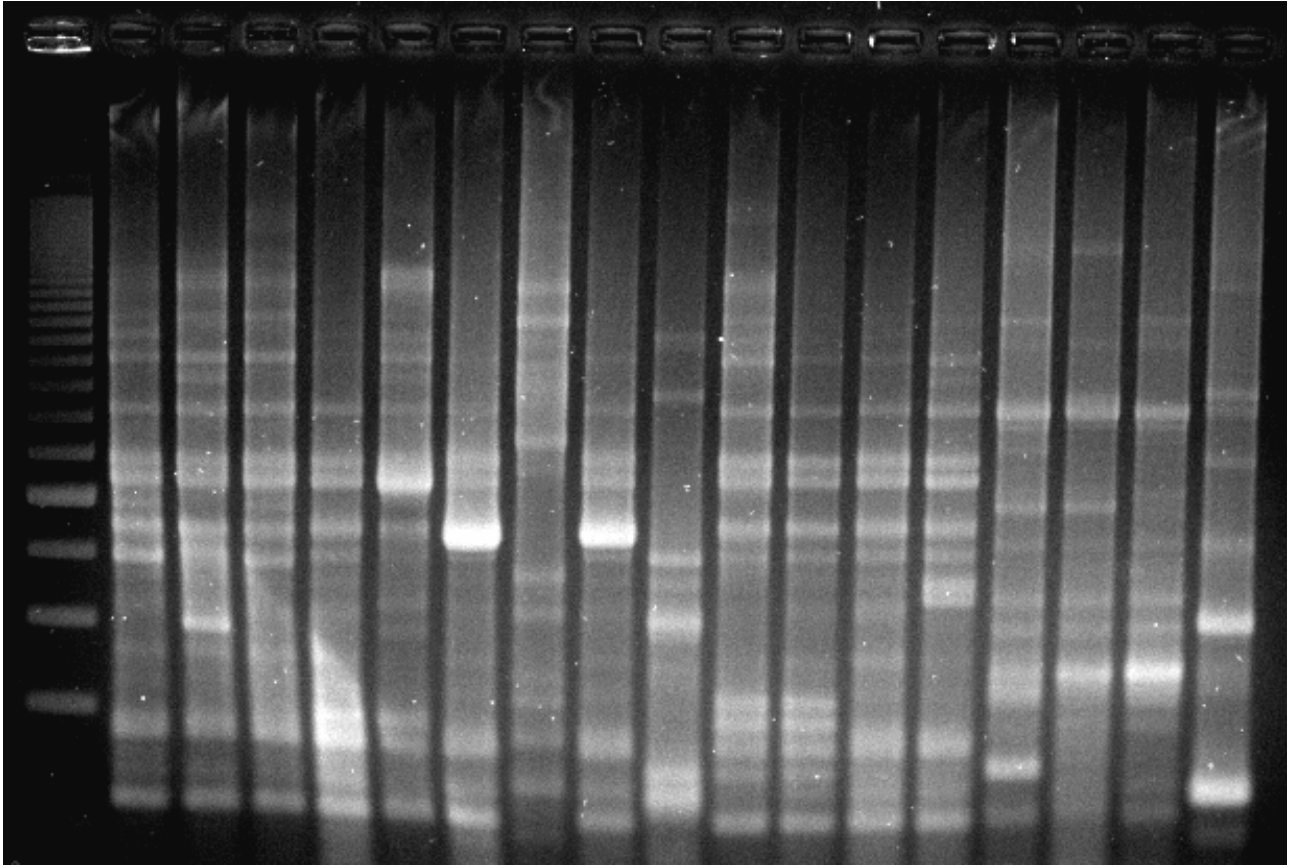


Fig. 2: rep-PCR profiles of *L. plantarum* and *L. fermentum* strains obtained with primer (GTG)₅ (in bold strains isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 ***L. plantarum* CBA-L150**
- 3 ***L. plantarum* CBA-L137**
- 4 ***L. plantarum* CBA-L138**
- 5 *L. plantarum* LMG 6907
- 6 *L. plantarum* LMG 21685
- 7 *L. plantarum* 3319
- 8 *L. plantarum* C21
- 9 *L. plantarum* P1730
- 10 *L. plantarum* F19
- 11 *L. plantarum* 69
- 12 *L. plantarum* P21021
- 13 *L. plantarum* 299V
- 14 *L. plantarum* LMG 17552
- 15 ***L. fermentum* CBA-L147**
- 16 ***L. fermentum* CBA-L146**
- 17 *L. fermentum* DSM20052
- 18 *L. fermentum* 1156

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

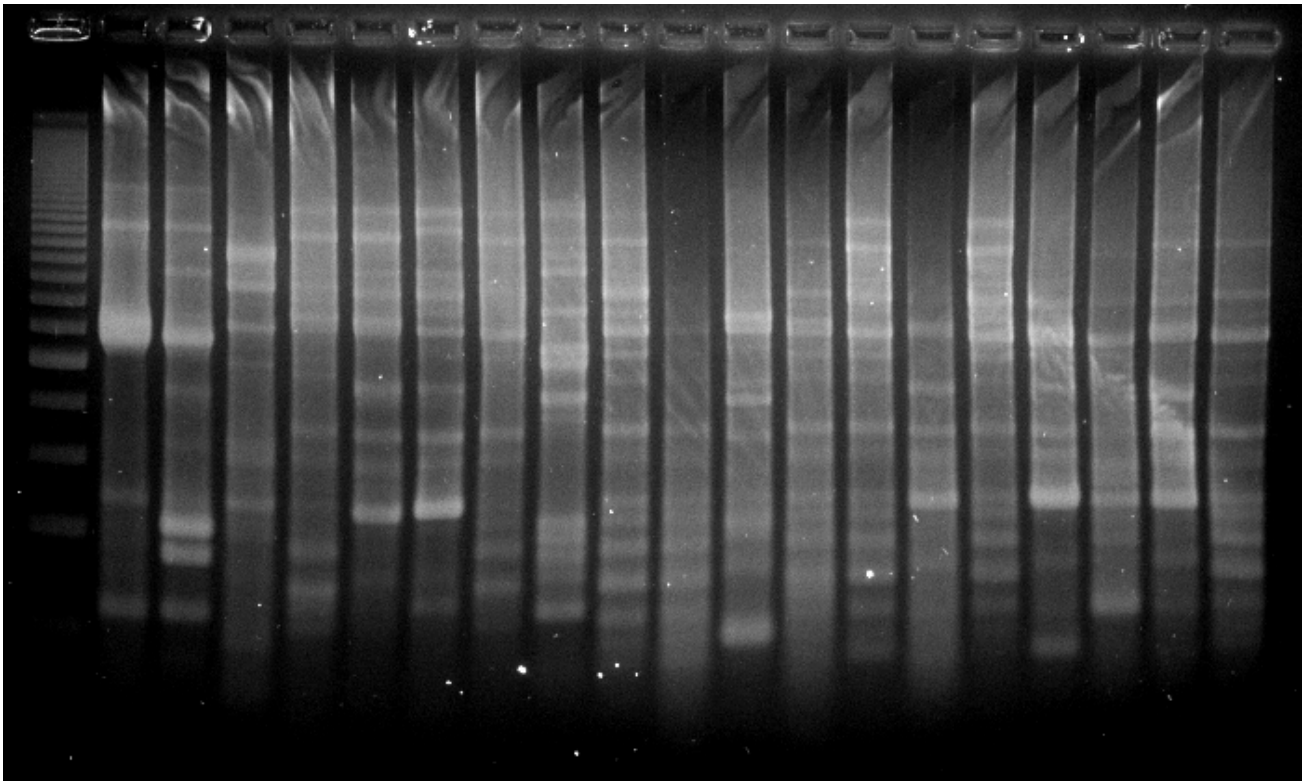


Fig. 3: rep-PCR profiles of *L. mucosae* and *L. rhamnosus* strains obtained with primer (GTG)₅ (in bold strains isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 ***L. mucosae* CBA-L149**
- 3 ***L. mucosae* CBA-L148**
- 4 *L. mucosae* LMG 19534
- 5 ***L. rhamnosus* CBA-L140**
- 6 ***L. rhamnosus* CBA-L142**
- 7 ***L. rhamnosus* CBA-L92**
- 8 ***L. rhamnosus* CBA-L143**
- 9 *L. rhamnosus* C48
- 10 *L. rhamnosus* ATCC 10863
- 11 *L. rhamnosus* DSM 20021
- 12 *L. rhamnosus* 1123
- 13 *L. rhamnosus* T12
- 14 *L. rhamnosus* GR-1
- 15 *L. rhamnosus* GG
- 16 *L. rhamnosus* commercial strain
- 17 *L. rhamnosus* ATCC 14957
- 18 *L. rhamnosus* R011
- 19 *L. rhamnosus* ATCC 53103
- 20 *L. rhamnosus* 5/5

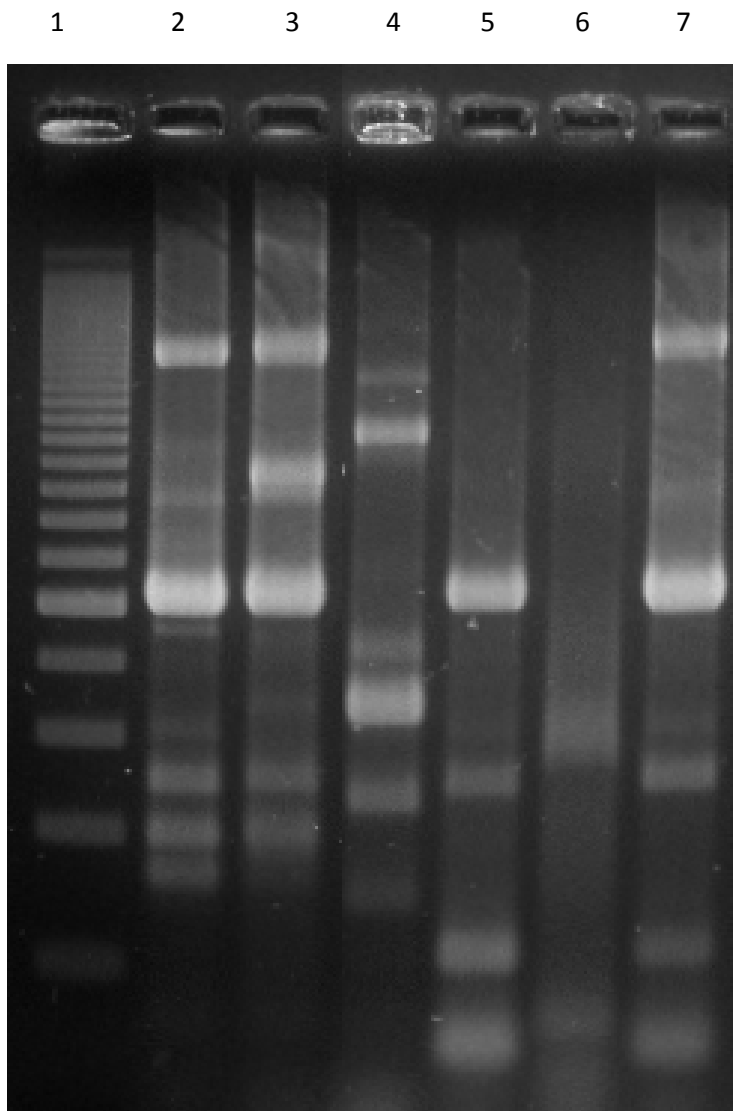


Fig. 4: rep-PCR profiles of *L. gasseri* obtained with primer (GTG)₅ (in bold the strain isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 *L. gasseri* CBA-L72**
- 3 *L. gasseri* CNBL 1403
- 4 *L. gasseri* DSM 20243
- 5 *L. gasseri* DSM 20077
- 6 *L. gasseri* DSM 20604
- 7 *L. gasseri* ATCC 19992

The Fig. 2 shows as the strains *L. plantarum* CBA-L150, *L. plantarum* CBA-L138 and *L. plantarum* 299V seem to share the same profile. The same situation, as shown in Fig. 3, occurs for strains of *L. rhamnosus* (*L. rhamnosus* CBA-L140 and *L. rhamnosus* CBA-L143 seem to have the same profile, as well as *L. rhamnosus* CBA-L142 and *L. rhamnosus* CBA-L92).

To ensure that these isolated are different strains it was set up a rep-PCR using the primers REP1R-Dt and REP2-Dt maintaining the same conditions described above. In Fig. 5 are shown

the rep-PCR profiles of lactobacilli for which it was made this further amplification because they were difficult to distinguish.

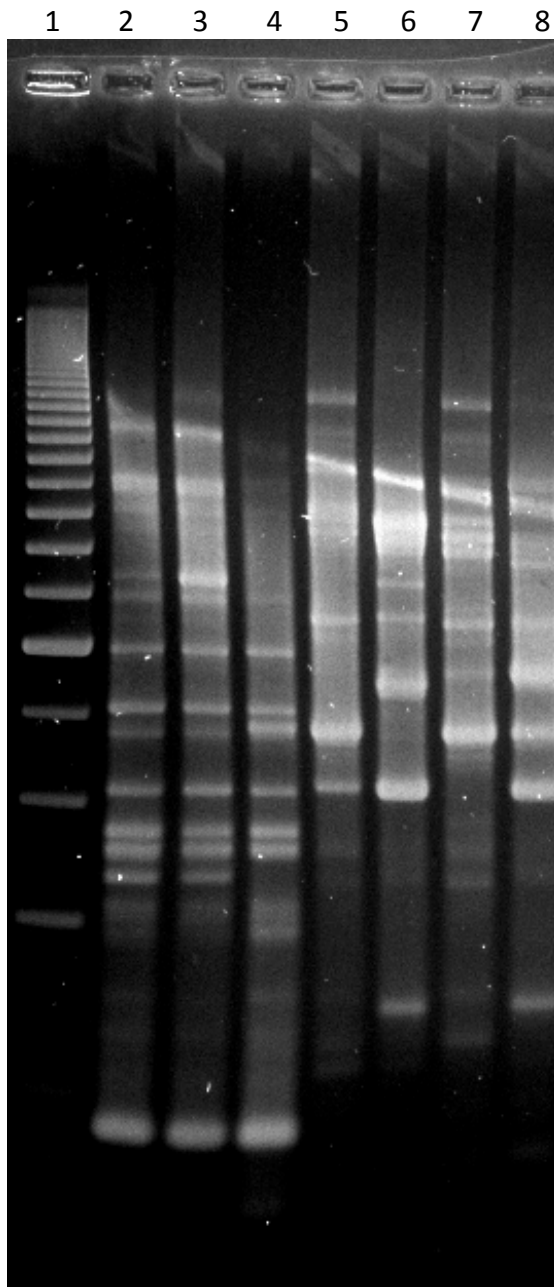


Fig. 5: rep-PCR profiles of *L. plantarum* and *L. rhamnosus* obtained with primers REP1R-Dt e REP2-Dt (in bold strains isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 ***L. plantarum* CBA-L150**
- 3 ***L. plantarum* CBA-L138**
- 4 *L. plantarum* 299V
- 5 ***L. rhamnosus* CBA-L140**
- 6 ***L. rhamnosus* CBA-L142**
- 7 ***L. rhamnosus* CBA-L143**
- 8 ***L. rhamnosus* CBA-L92**

As shown in Fig. 5 the strains *L. plantarum* CBA-L150 and *L. plantarum* CBA-L138 have the same profile (the rep-PCR set using primers REP1R-Dt e REP2-Dt confirms the result of the amplification with primer (GTG)₅), most likely it is the same strain of *L. plantarum*. The final PCR reaction instead confirms that strains of *L. rhamnosus* isolated are different, in fact, they have a different rep-PCR profile (Fig. 5).

For all other lactobacilli isolated it has not been necessary repeat the rep-PCR because they were well discriminated and especially different from each other and from other strains of the same species already present on the market or in the bacterial collections (Fig. 1, 2, 3, 4).

For identification of bifidobacteria we decided that rep-PCR using the BOXA1R primer was the best method because, as described by Masco *et al.*, 2003, this primer has the highest discriminatory power.

The Fig. 6 and 7 show rep-PCR profiles of strains of *B. pseudocatenulatum*, *B. bifidum*, *B. thermoacidophilum*, *B. lactis* (Fig. 6) and strains of *B. breve*, *B. adolescentis*, *B. longum* (Fig. 7).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

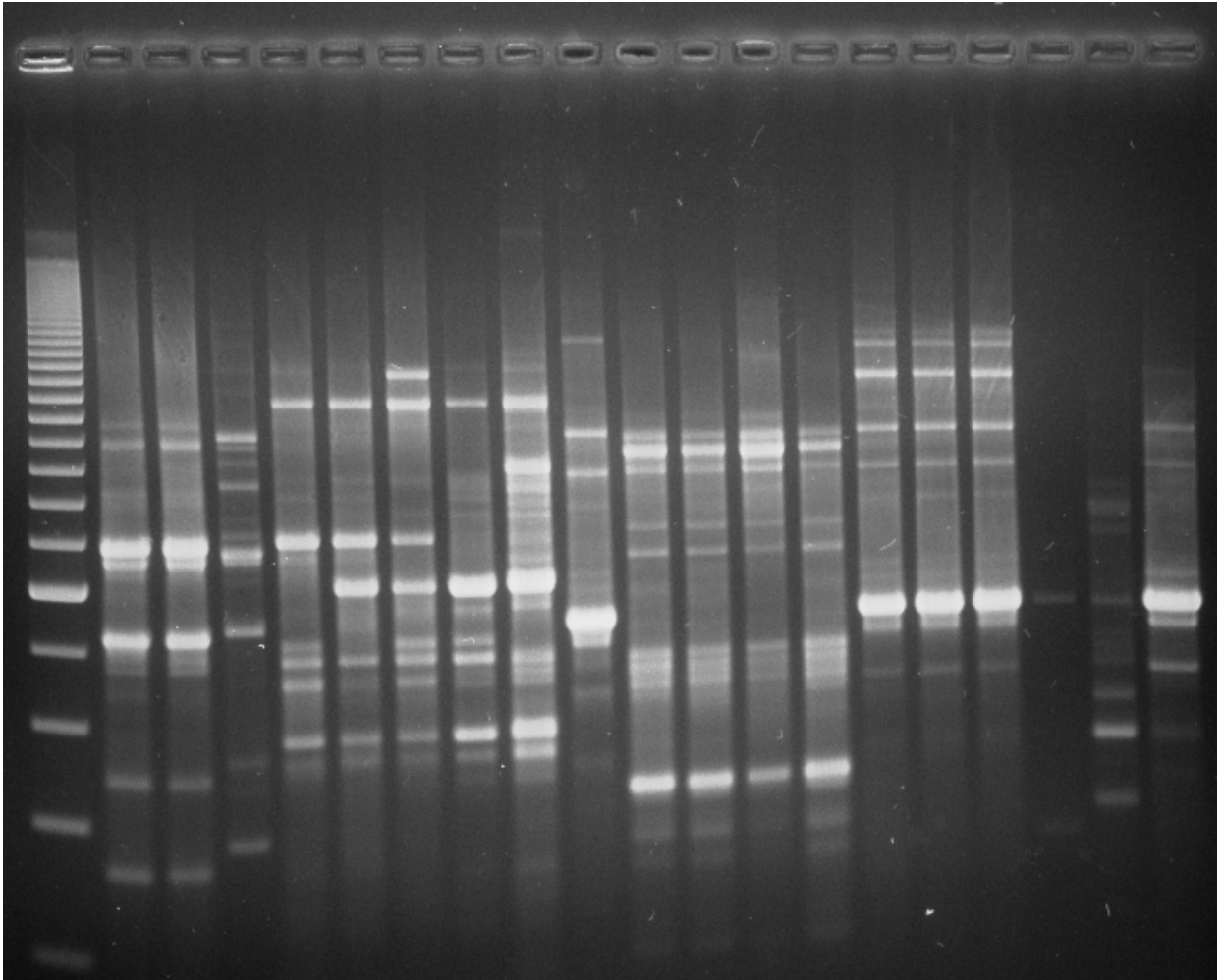


Fig. 6: rep-PCR profiles of *B. pseudocatenulatum*, *B. bifidum*, *B. thermoacidophilum* and *B. lactis* obtained with primer BOXA1R (in bold strains isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 ***B. pseudocatenulatum* CBA-B152**
- 3 ***B. pseudocatenulatum* CBA-B149**
- 4 *B. pseudocatenulatum* LMG 10505
- 5 ***B. bifidum* CBA-B98**
- 6 ***B. bifidum* CBA-B150**
- 7 ***B. bifidum* CBA-B151**
- 8 *B. bifidum* LMG 11041
- 9 *B. bifidum* commercial strain
- 10 *B. bifidum* 1070
- 11 ***B. thermoacidophilum* CBA-B86**
- 12 ***B. thermoacidophilum* CBA-B106**
- 13 ***B. thermoacidophilum* CBA-B85**
- 14 ***B. thermoacidophilum* CBA-B84**
- 15 *B. lactis* CBA-B122
- 16 ***B. lactis* CBA-B132**
- 17 *B. lactis* BL04
- 18 *B. lactis* BB-12
- 19 *B. lactis* W11
- 20 *B. lactis* commercial strain

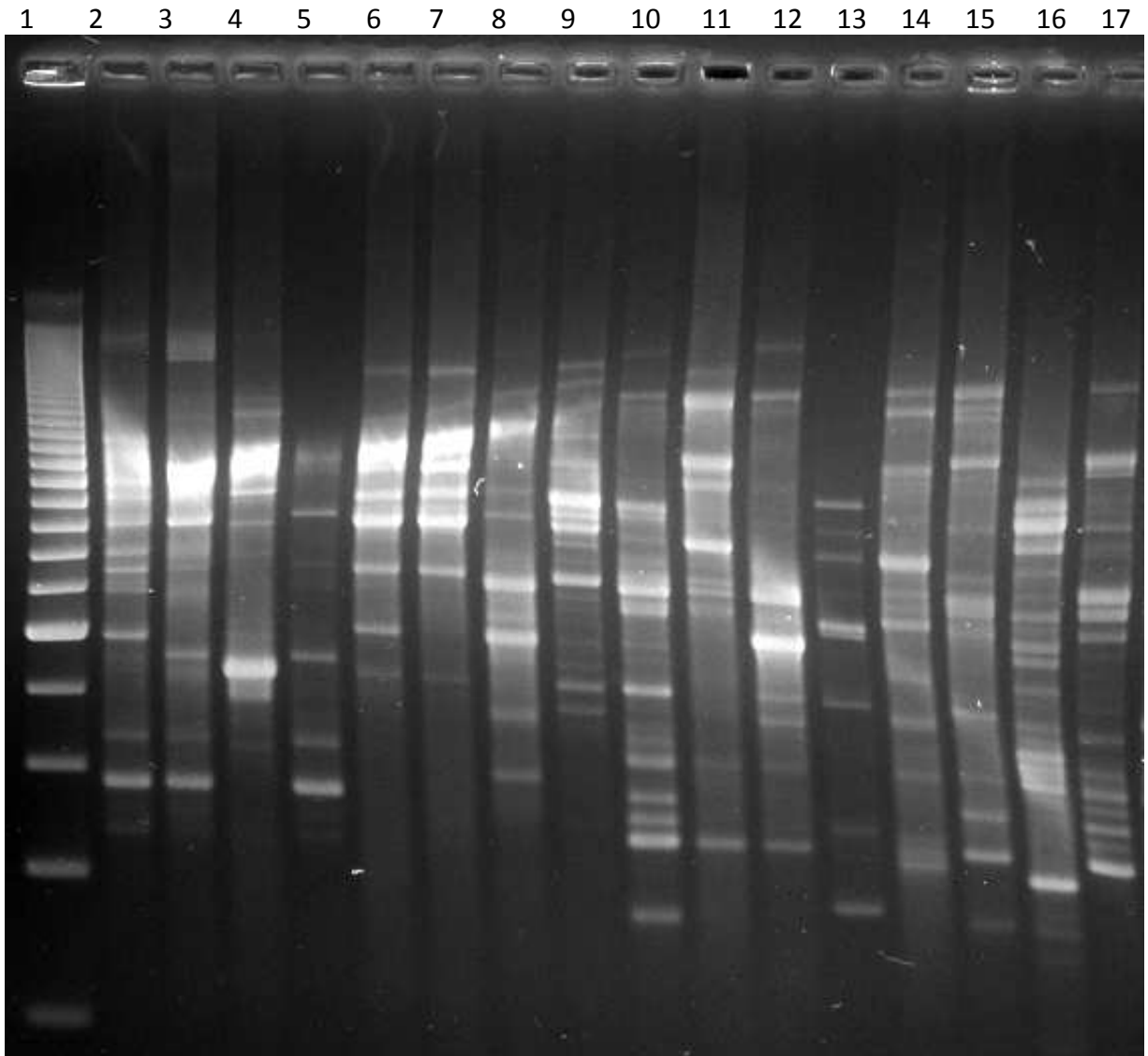


Fig. 7: rep-PCR profiles of *B. breve*, *B. adolescentis*, *B. longum* obtained with primer BOXA1R (in bold strains isolated in this study)

- 1 Molecular weight marker 200 bp
- 2 ***B. breve* CBA-B104**
- 3 ***B. breve* CBA-B171**
- 4 *B. breve* LMG 13208
- 5 *B. breve* DSM 16604
- 6 ***B. adolescentis* CBA-B83**
- 7 ***B. adolescentis* CBA-B81**
- 8 *B. adolescentis* 1146
- 9 *B. adolescentis* DSM 20083
- 10 ***B. longum* CBA-B128**
- 11 ***B. longum* CBA-B170**
- 12 *B. longum* LMG 13197
- 13 *B. longum* LMG 21814
- 14 *B. longum* DSM 20088
- 15 *B. longum* W11
- 16 *B. longum* DSM 16603
- 17 *B. longum* BL 999

As shown in Fig. 6, *B. pseudocatenulatum* CBA-B152 and *B. pseudocatenulatum* CBA-B149 have the same rep-PCR profile, the same situation also occurs for strains CBA-B86, CBA-B106, CBA-B84 of *B. thermoacidophilum*. Moreover *B. lactis* CBA-B122 and CBA-B132 have the same rep-PCR profile and equal to that of the commercial strain *B. lactis* BL04.

So it has been repeated rep-PCR amplification of *B. lactis* CBA-B122, CBA-B132 and *B. lactis* BL04 using the primer (GTG)₅ (Figure 8).

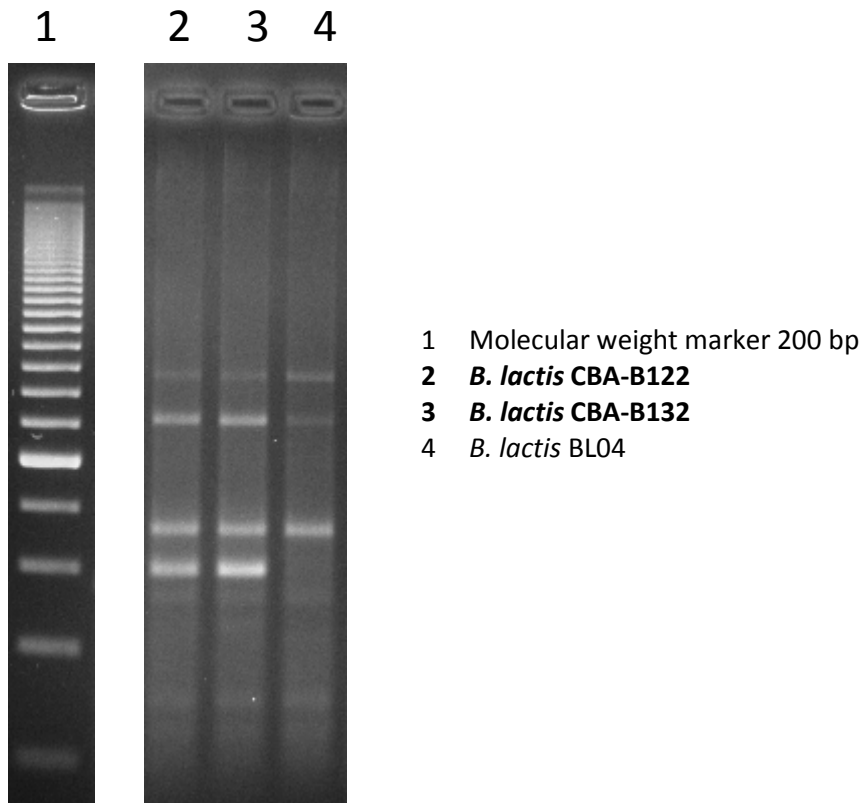


Fig. 8: rep-PCR profiles of *B. lactis* strains obtained with primer (GTG)₅ (in bold strains isolated in this study)

The latter figure shows that the commercial strain *B. lactis* BL04 is different from the two strains of *B. lactis* isolated in this work that, however, are resulted equal to each other even after this amplification reaction.

Effects of bile salts on viability

The effect of bile salts on the viability of selected *Lactobacillus* and *Bifidobacterium* is presented respectively in Table 1 and 2. Comparison of the ability of the strains to grow in liquid medium with and without bile salts revealed some variation among strains.

Tables 1 and 2 show, for each bacterial strain, the OD values reached in presence and absence of bile salts in the culture medium. Particularly, T0: represents the OD values obtained at time zero, T16 is the OD carried out after 16 hours of incubation of the culture medium inoculated at 37°C and T24 is the OD value after 24 hours incubation of the same culture medium inoculated at 37°C.

	T0		T16		T24	
	MRS	MRS + Bile salts	MRS	MRS + Bile salts	MRS	MRS + Bile salts
<i>L. paracasei</i> LMG S-27487	0,089	0,107	1,808	1,089	1,376	1,282
<i>L. paracasei</i> CBA-L101	0,068	0,081	1,232	0,497	1,703	0,740
<i>L. paracasei</i> CBA-L145	0,087	0,101	1,897	0,995	1,969	1,186
<i>L. paracasei</i> CBA-L144	0,058	0,054	2,012	0,961	2,096	1,086
<i>L. plantarum</i> CBA-L137	0,094	0,108	1,955	1,119	2,035	1,395
<i>L. plantarum</i> CBA-L138	0,100	0,118	2,014	1,550	2,095	1,641
<i>L. plantarum</i> CBA-L150	0,072	0,070	2,045	1,787	2,108	1,840
<i>L. rhamnosus</i> CBA-L92	0,091	0,109	1,867	0,800	1,952	1,233
<i>L. rhamnosus</i> CBA-L142	0,101	0,116	2,050	1,386	2,124	1,522
<i>L. rhamnosus</i> CBA-L143	0,041	0,042	1,836	0,778	1,855	0,874
<i>L. rhamnosus</i> CBA-L140	0,097	0,113	1,918	1,051	1,962	1,121
<i>L. acidophilus</i> CBA-L63	0,076	0,091	1,664	1,330	1,695	1,620
<i>L. rhamnosus</i> GG	0,108	0,102	2,064	1,332	2,139	1,629
<i>L. gasseri</i> CBA-L72	0,034	0,029	1,835	0,972	1,875	1,129
<i>L. gasseri</i> CBA-L100	0,026	0,023	1,715	0,215	1,760	0,186
<i>L. crispatus</i> CBA-L154	0,021	0,022	1,129	0,070	1,525	0,075
<i>L. crispatus</i> CBA-L157	0,031	0,028	1,180	0,122	1,514	0,126
<i>L. crispatus</i> CBA-L158	0,028	0,021	1,061	0,267	1,472	0,271
<i>L. jensenii</i> CBA-L160	0,034	0,024	1,976	0,074	2,069	0,051
<i>L. fermentum</i> CBA-L146	0,027	0,028	2,046	1,622	2,011	1,713
<i>L. fermentum</i> CBA-L147	0,048	0,050	1,951	1,682	1,957	1,768
<i>L. mucosae</i> CBA-L148	0,013	0,010	1,117	1,342	1,539	1,282
<i>L. mucosae</i> CBA-L149	0,015	0,008	1,191	0,909	1,457	0,931
<i>L. vaginalis</i> CBA-L88	0,014	0,037	1,427	1,185	1,595	1,247

Table 1: ability of the lactobacilli to grow in the presence of bile salts evaluated by mean of OD measurements

As shown in Table 1, strains *L. gasseri* CBA-L100, *L. crispatus* CBA-L154, *L. crispatus* CBA-L157, *L. crispatus* CBA-L158 and *L. jensenii* CBA-L160 are not able to grow in presence of bile salts; in fact the value of optical density of the culture medium inoculated and containing the salts of bile after 24 hours of incubation at 37°C appears to be not significantly different from that read at time zero, testimony of a non-bacterial growth. Potentially a strain could survive to the bile salts even if it is not able to grow and therefore, in this case, the optical density would still remain unchanged. For this reason, for the bacterial strains for which the optical density is not

changed it was determined bacterial viability and I have shown that the latter was decreased despite the optical density has remained the same.

The other lactobacilli showed however a capacity of growth in the presence of bile salts, this means that 0,3% of bile salts exerted only a small inhibitory effect on these strains and that these strains studied showed relatively a good resistance to bile salts. *L. acidophilus* CBA-L63 is the most resistant strain with about the same OD values in presence and absence of bile salts.

Given the fact that the growth of bifidobacteria is more slow, for them, the absorbance was also measured after 48 and 72 hours of incubation of culture medium inoculated at 37°C in anaerobic conditions.

	T0		T16		T24		T48		T72	
	MRS CYS	MRS CYS + Bile salts	MRS CYS	MRS CYS + Bile salts	MRS CYS	MRS CYS + Bile salts	MRS CYS	MRS CYS + Bile salts	MRS CYS	MRS CYS + Bile salts
<i>B. longum</i> CBA-B170	0,017	0,021	0,022	0,029	0,021	0,025	0,016	0,619	0,655	0,621
<i>B. bifidum</i> CBA-B151	0,041	0,033	0,040	0,032	0,045	0,006	0,056	0,101	1,535	0,272
<i>B. thermoacidophilum</i> CBA-B85	0,075	0,085	1,662	0,197	1,972	0,211	2,100	0,263		
<i>B. thermoacidophilum</i> CBA-B86	0,024	0,028	0,025	0,033	1,544	0,078	2,029	0,545		
<i>B. thermoacidophilum</i> CBA-B106	0,008	0,000	0,017	0,002	0,025	0,059	1,829	0,076		
<i>B. breve</i> CBA-B104	0,022	0,028	0,070	0,044	0,133	0,044	0,959	0,050	1,335	0,061
<i>B. breve</i> CBA-B171	0,014	0,011	0,014	0,014	0,120	0,130	1,463	0,296	1,402	0,377
<i>B. lactis</i> CBA-B122	0,030	0,007	0,083	0,040	0,110	0,126	1,274	0,280	1,492	0,267
<i>B. lactis</i> CBA-B132	0,024	0,035	0,072	0,101	0,065	0,118	0,491	0,499	1,467	0,537

Table 2: ability of bifidobacteria to grow in the presence of bile salts

Table 2 shows that all bifidobacteria tested have a poor ability to grow in the presence of bile salts; in fact the value of optical density of the culture medium inoculated and containing bile salts even after 72 hours of incubation at 37°C appears to be not significantly different from that read at time zero (OD always less than one), evidence of a poor bacterial growth. In the case of the strains of *B. thermoacidophilum* we have not been carried out the reading of the optical density at T72 hours because they are able to reach the "peak" of growth already after 48 hours of incubation.

Effects of gastric acidity on viability

Preliminary experiments were conducted to determine the degree of acid resistance exhibited by *Lactobacillus* and *Bifidobacterium* strains isolated because the ability of the strains to survive in the presence of pepsin is an accurate indicator of the ability of the strains to survive in the stomach. The results were compared with the resistance of *L. rhamnosus* GG, strain resistant to the acidity (data presented in the section "supplementary material"). Analysis of the results showed that many of the lactobacilli isolated had a good degree of resistance to acidity and this suggest the possibility of using these strains as probiotics because they are able to pass through the stomach and reach the intestine to exert their beneficial effects. Bifidobacteria, however, were less acid resistance than lactobacilli especially if put in contact for a long time with the solution of pepsin.

Antibiotic Susceptibility Testing

Micro-dilution method was used to assess the susceptibility of the LAB strains isolated to 9 antibiotics, ampicillin (Sigma-Aldrich, Germany), vancomycin (Sigma-Aldrich, Germany), streptomycin (Sigma-Aldrich, Germany), gentamicin (Sigma-Aldrich, Germany), kanamycin (Sigma-Aldrich, Germany), erythromycin (Sigma-Aldrich, Germany), clindamycin (Sigma-Aldrich, Germany), tetracycline (Sigma-Aldrich, Germany) and chloramphenicol (Sigma-Aldrich, Germany). The MIC values obtained are presented in Tables 3 (lactobacilli) and 4 (bifidobacteria). The strains were classified as resistant or sensitive to a specific antibiotic according to the cut-off values proposed by EFSA and listed in Tables 3 and 4.

	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>L. paracasei</i> LMG S-27487	2		16	128	64	0,25	0,25	1	4
<i>L. paracasei</i> CBA-L101	0,5		<4	<8	16	<0,25	1	<2	8
<i>L. paracasei</i> CBA-L145	8		8	128	32	0,25	0,25	2	4
<i>L. paracasei</i> CBA-L144	4		8	64	32	0,5	0,125	1	8
B.P. EFSA (<i>L. casei/paracasei</i>)	4	n.r.	32	64	64	1	1	4	4
	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>L. reuteri</i> CBA-L167	16		4	128	32	0,5	0,064	>64	64
<i>L. reuteri</i> CBA-L168	4		2	64	32	>8	>16	>64	>64
B.P. EFSA (<i>L. reuteri</i>)	2	n.r.	8	64	64	1	1	16	4
	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>L. plantarum</i> CBA-L137	1		1	16	8	0,25	0,032	16	2
<i>L. plantarum</i> CBA-L138	1		1	32	8	0,5	0,064	8	4
<i>L. plantarum</i> CBA-L150	1		2	32	16	1	0,25	32	4
B.P. EFSA (<i>L. plantarum/pentosus</i>)	2	n.r.	16	64	n.r.	1	2	32	8
	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>L. rhamnosus</i> CBA-L92	<0,5		8	64	16	<0,25	0,5	<2	2
<i>L. rhamnosus</i> CBA-L142	4		8	64	16	0,25	0,5	2	8
<i>L. rhamnosus</i> CBA-L140	4		16	128	32	0,5	0,5	1	8
<i>L. rhamnosus</i> CBA-L143	2		2	64	4	0,5	0,5	2	8
B.P. EFSA (<i>L. rhamnosus</i>)	4	n.r.	16	64	32	1	1	8	4
	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>L. acidophilus</i> CBA-L63	<0,5	<0,5	<4	<8	16	<0,25	1	<2	2
<i>L. gasseri</i> CBA-L72	<0,5	1	4	16	8	<0,25	4	4	8
<i>L. gasseri</i> CBA-L100	<0,5	1	8	128	32	<0,25	4	4	4
<i>L. crispatus</i> CBA-L154	4	1	4	64	2	0,125	0,032	1	2
<i>L. crispatus</i> CBA-L155	8	1	16	128	32	0,25	0,032	1	4
<i>L. crispatus</i> CBA-L156	4	1	16	256	16	0,125	0,25	1	2
<i>L. crispatus</i> CBA-L157	8	1	16	128	16	0,125	0,032	1	2
<i>L. crispatus</i> CBA-L158	8	1	8	32	16	0,125	0,032	1	2
<i>L. crispatus</i> CBA-L159	8	1	32	256	16	0,25	0,064	1	2
<i>L. jensenii</i> CBA-L160	0,5	2	4	16	2	0,125	0,25	1	1
<i>L. amylovorus</i> CBA-L164	4	>128	16	128	32	0,5	0,5	1	8
B.P. EFSA (<i>L. acidophilus</i> group)	1	2	16	64	16	1	1	4	4
	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>L. fermentum</i> CBA-L90	1		8	>128	128	<0,25	4	32	8
<i>L. fermentum</i> CBA-L146	2		1	32	16	0,25	0,032	4	4
<i>L. fermentum</i> CBA-L147	2		2	64	32	1	0,032	4	4
<i>L. fermentum</i> CBA-L163	2		2	32	32	0,5	0,032	8	8
<i>L. fermentum</i> CBA-L161	1		32	128	64	0,5	0,064	4	16
<i>L. fermentum</i> CBA-L162	2		4	128	64	0,5	0,032	4	4
<i>L. mucosae</i> CBA-L148	1		1	4	16	0,125	0,032	4	8
<i>L. mucosae</i> CBA-L149	1		2	16	16	0,25	0,032	16	8
<i>L. vaginalis</i> CBA-L88	<0,5		<4	16	64	<0,25	<0,25	>32	2
B.P. EFSA (<i>Lactobacillus obligate heterofermentative</i>)	2	n.r.	16	32	64	1	1	8	4

Table 3: MIC distributions for 9 antibiotics of lactobacilli isolates (in bold cut-off values defined by EFSA, 2012 for lactobacilli)

AMP = ampicillin, VAN = vancomycin, GEN = gentamicin, KAN = kanamycin, STR = streptomycin, ERY = erythromycin, CLI = clindamycin, TET = tetracycline, CHL = chloramphenicol

	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
<i>B. pseudocatenulatum</i> CBA-B149	0,125	0,5	128	512	128	0,25	0,064	1	2
<i>B. bifidum</i> CBA-B150	0,25	0,5	16	64	4	0,125	0,032	0,25	1
<i>B. lactis</i> CBA-B122	0,5	0,5	256	1024	128	0,25	0,064	16	2
<i>B. pseudocatenulatum</i> CBA-B152	0,125	0,5	64	256	64	0,25	0,064	1	2
<i>B. adolescentis</i> CBA-B81	0,5	0,5	128	512	128	0,125	0,032	0,5	4
<i>B. bifidum</i> CBA-B98	0,25	0,5	64	64	>256	0,125	0,125	0,5	1
<i>B. lactis</i> CBA-B132	0,5	0,5	>256	1024	256	0,25	0,064	16	2
<i>B. bifidum</i> CBA-B151	0,125	0,5	64	256	16	0,125	0,125	0,5	1
<i>B. breve</i> CBA-B104	2	1	64	512	32	0,25	0,032	0,5	2
<i>B. adolescentis</i> CBA-B83	0,5	0,5	128	512	32	0,125	0,032	1	1
<i>B. breve</i> CBA-B171	1	0,5	32	128	>256	0,125	0,032	2	0,5
<i>B. longum</i> CBA-B170	2	1	64	1024	>256	0,25	0,064	16	1
<i>B. thermoacidophilum</i> CBA-B84	0,125	0,5	16	64	32	0,125	0,125	64	2
<i>B. longum</i> CBA-B128	1	0,5	16	16	32	0,125	0,032	0,5	0,5
<i>B. thermoacidophilum</i> CBA-B86	0,25	0,5	64	256	32	0,125	0,125	64	2
<i>B. thermoacidophilum</i> CBA-B106	0,25	0,5	16	512	16	0,125	0,064	32	2
<i>B. thermoacidophilum</i> CBA-B85	0,064	0,5	16	64	16	0,125	0,032	32	2
B.P. EFSA (<i>Bifidobacterium</i>)	2	2	64	n.r.	128	1	1	8	4

Table 4: MIC distributions for 9 antibiotics of bifidobacteria isolates (in bold cut-off values defined by EFSA, 2012 for bifidobacteria)

All of the lactobacilli isolates were found to be susceptible to erythromycin, except *L. reuteri* CBA-L168 that had a very high value of MIC for this antibiotic. Another antibiotic to which all lactobacilli isolates were sensitive is gentamicin: only two strains in fact have, for this antibiotic, a MIC twice the breakpoint. High level of resistance to ampicillin were observed among *L. reuteri* and lactobacilli that belong to the *L. acidophilus* group, while almost half of lactobacilli isolates tend to be resistant to kanamycin. Another antibiotic to which about 50% of lactobacilli were resulted resistant is chloramphenicol, with the exclusion of bacteria of the *L. acidophilus* group for which the incidence of resistance is low and the strains of *L. plantarum* that were resulted sensitive to chloramphenicol. Low levels of resistance to streptomycin, clindamycin and tetracycline were observed among all lactobacilli (12,1% for streptomycin and clindamycin and 15,2% for tetracycline).

Among bifidobacteria, the antibiotic resistance to tetracycline is resulted very common and affects 41.2% of the isolates. Other antibiotics for which occurred resistances are gentamicin and streptomycin, affecting respectively 29.4% and 23.5% of isolates. All bifidobacteria isolates were found to be sensitive to ampicillin, vancomycin, kanamycin, erythromycin, clindamycin and chloramphenicol.

Conclusions

In the last few years interest in the physiology and genetics of LAB species has increased greatly, and this is mainly due to the fact that these bacteria are increasingly used as starters and they are a key in the market of probiotics. LAB possess a lot of desirable properties and this represents an important value in the production of fermented products and food supplements. However, the beneficial effects of probiotics must be demonstrated primarily through *in vitro* tests; in this work we have done a careful screening of LAB taking into consideration some criteria generally used to select and assess potential gut probiotic bacteria. More precisely, we focused our attention on the topic of safety of the bacterial strain, a fundamental requirement for use in humans and on the ability of the strains to live in conditions similar to those of the human gastrointestinal tract that is in the presence of low values of pH and bile salts. The criteria used in our study to identify and characterize new potential gut probiotic bacteria resulted in the identification of a small number of lactobacilli and bifidobacteria that do not show antibiotic resistance and that are capable of resisting the effects of bile and low pH *in vitro*.

So, in this study we showed that the adoption of these criteria for *in vitro* selection of gut probiotic bacteria allows the isolation of strains that are certainly safe for use in humans and potentially capable to exert their effect in the gastrointestinal tract through the introduction of these probiotic strains in food supplements.

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Chapter 3 – Spray-drying encapsulation of probiotics for ice-cream application

Spray-drying encapsulation of probiotics for ice-cream application

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Ice-cream is generally considered as a nutritive food, representing an interesting vehicle for delivering beneficial microorganisms to consumers. Many ice-cream makers use industrial dry bases for its preparation. The aim of this study was to investigate the possibility of producing spray-dried probiotic formulations to be used together with commercial ice-cream bases.

A commercial cream base was selected for the study and, according to its composition and literature, two different formulations were investigated. Formulation A (FA) consisted in 46 % commercial skim milk powder, 24 % anhydrous glucose, 28 % maltodextrin (Maltrin® 40) and 2 % sodium alginate. Formulation B (FB) contained prebiotic inulin fiber (Fibruline® instant) instead of Maltrin®. Powders were obtained with a lab-scale spray dryer dissolving each formulation in water at a 10 % w/v, with a 6 mL/min flow rate, 150 °C inlet temperature and increasing feed volume (100, 200, 300, 400 mL). The process gave a total dry solids yield ranging from 62.07 % (with 100 mL feed volume) to 58.14 % (with 400 mL) for FA, and from 65.55 % to 59.46 % for FB. Powder a_w was always < 0.3 .

The formulations were used for encapsulation of a strain of *Lactobacillus paracasei*. Considering a recommended probiotics minimum daily intake of 10^9 CFU, a 100 g ice-cream serving, a 5 % substitution of the commercial base with the probiotic-enriched base and supposing no vital loss during the process, the cellular feed concentration for the spray-drying trials was calculated. However, in the presence of probiotics, FA could not be spray dried, while probiotic-FB was obtained with a 50.89 % yield, 0.42 a_w and 82 % cell mortality. Ice-cream was finally prepared in a domestic ice-cream maker using the probiotic-enriched FB after the pasteurization step and before the 12 h maturation step which caused limited additional mortality.

Introduction

Dairy products with incorporated probiotic bacteria are gaining popularity and the probiotics comprise approximately 65 % of the world functional food market (Akalin and Erişir, 2008). Although the application of probiotics in cheeses and especially in fermented milks has been widely explored in the literature, ice-cream is a relatively innovative and apparently suitable matrix for delivering probiotics in human diet because of its pleasant taste and attractive texture. Furthermore, ice-cream is highly accepted product by children, adolescents, and adults, as well as by the elderly public and even though it is more consumed in the summer due

to its rather refreshing features, some people have the habit of consuming it throughout the year (Cruz et al., 2009).

Probiotics are live microorganisms that, if administered in adequate amounts, confer health benefits on the host. Therefore, probiotic food can be considered as functional food containing viable probiotic microorganisms which must be able to survive in the gastrointestinal tract. International guide lines, such as the Italian Health Ministry Guide lines on probiotics and prebiotics (2013), indicate a recommended daily intake of 10^9 vital cells (as colony forming units, CFU), even though the exact dosage depends on the selected probiotic, probiotic blend and desired clinical outcome.

Many “hand-made” ice-cream makers use industrial dry bases for product preparation. The aim of this study was, then, to investigate the possibility of producing dried probiotic formulations to be easily used together with commercial ice-cream bases.

Encapsulation is the envelopment of small solid particles, liquid droplets or gases in a coating. It can be successfully applied to entrap natural compounds, like essential oils or vegetal extracts containing antioxidant polyphenols (Spigno et al., 2013) or pigments (De Marco et al., 2013). Microencapsulation (final particle size 1–1000 nm) can also be used to preserve lactic acid bacteria, both starters and probiotics, in food and during the passage through the gastrointestinal tract, contributing to the development of new functional foods (Nazzaro et al., 2012). Probiotic cell concentrates often need to be stored over longer periods prior to food manufacture and ingestion, therefore sometimes they are dried after production. The most common procedure is to produce hydrogel-based microcapsules by extrusion or emulsification processes and, then, freeze dry them. An alternative method to achieve capsule-building and drying in a single step is spray-drying, which is a routine process in the food industry to convert liquids (Heidebach et al., 2012), including also particulate fluids (Oi et al., 2013), into dry powders. A variety of materials can be used as carrier for probiotics encapsulation, such as arabic gum, alginates, maltodextrins, pectins, milk proteins, starch and chitosan among the others. In particular, reconstituted skim milk (RSM) has shown a positive effect on the cellular survival after spray-drying in combination with prebiotics, such as inulin (Fritzen-Freire et al., 2012). Also glucose addition has reduced spray-drying mortality of probiotics (Ying et al., 2012). Considering the above reviewed literature and having in mind a concept of tailor made encapsulation process, according to which the encapsulating materials should be selected based on the composition of the target food application, this work investigated the possibility

of encapsulating probiotic cultures in a formulation with a composition the closest possible to that of the ice-cream base. First, the influence of the use of maltodextrins rather than inulin in combination with RSM, glucose and alginate, and of the feeding volume on the process solids yield and final powder water activity was evaluated. Then, the two tested formulations (with maltodextrins or inulin) were applied for probiotics encapsulation to assess process feasibility and cellular survival. Finally, spray-dried cultures were employed for ice-cream preparation.

Materials and Methods

Preparation of modified ice-cream base formulations

A commercial base for full cream ice-cream preparation (Base Tuttapanna 100, Pernigotti, Italy) was used as reference encapsulating material and for ice-cream preparation. Base ingredients, in decreasing order, are: powder skim milk, powder full cream, dextrose, emulsifiers (E472, E471, E473), anhydrous glucose syrup, maltodextrins, concentrated milk proteins, stabilizers (E401, E410, E412), vanillin, natural flavour.

The commercial base and two different formulations were tested for spray-drying:

Formulation A (FA): 46 % commercial skim milk powder (Regilait), 24 % anhydrous glucose (Carlo Erba, Italy), 28 % maltodextrin (Maltrin[®] 40, Grain Processing Corporation GPC, kindly supplied by LEHVOSS Italia S.R.L.) and 2 % sodium alginate (Carlo Erba, Italy).

Formulation B (FB): as FA but with 28 % of prebiotic inulin fiber (Fibruline[®] instant, kindly provided by COSUCRA, VICTA Food & Trade, Italy) instead of Maltrin[®].

All the used materials were characterised for moisture content (by drying at 105 °C until constant weight).

Powders were obtained with a lab-scale spray dryer (Büchi Mini Spray dryer B-290) dissolving each formulation at a 10 % w/v in water, with a 6 mL/min flow rate, 150 °C inlet temperature and increasing feed volume (100, 200, 300, 400 mL). Trials were carried out in triplicate. For each test, outlet temperature was recorded and the collected powder was weighted and analysed for moisture content (by drying at 105 °C until constant weight) and a_w (AquaLab Dew Point Water Activity Meter 4TE).

The total percent dry solids yield was calculated as the ratio of outlet dry matter to the inlet dry matter content.

Preparation of probiotics enriched formulations

For the preparation of the probiotic functionalised ice-cream the commercial base was substituted with a 5 % of a modified base (as FA or FB) enriched with probiotics (Figure 1). The required cellular concentration for the solutions to be spray-dried was calculated based on the following data or hypotheses:

No vitality loss occurs during spray-drying;

A final ice-cream with a 10^8 CFU/g is desired so that a 100 g serving would guarantee the recommended minimum 10^9 CFU daily intake;

The ice-cream yield is 1350 g / 100 g of base (as evaluated in preliminary trials);

Spray-drying feeding solution has a 10 % w/v solids of formulation ingredients.

Considering the process yields obtained with FA and FB and the required powder amounts for analysis and ice-cream preparation, it was established to spray-dry 600 mL of solution (Figure 1). Trials were carried out in duplicate and process evaluated as reported in section 2.1.

Probiotic strain *Lactobacillus paracasei* LMG S-27487 was cultured in broth of De Man, Rogosa & Sharp (MRS) medium (Difco, US) at 37 °C in microaerophilic conditions for 18 h. A concentrated culture pellet from 1500 mL MRS was harvested by centrifugation at 8.000 rpm for 15 min, washed twice with sterile distilled saline and finally resuspended in 200 mL of water containing the base. The CFU concentration was evaluated and the suspension was stored at 4 °C until being diluted to 600 mL for spray-drying.

Viable probiotic cells in the suspension and powder were enumerated by decimal dilutions into maximum recovery diluent (MRD) (Difco, US) and plated onto agar MRS medium. Plates were anaerobically incubated at 37 °C for 72 h. The number of colonies for two parallel plates was counted from a dilution yielding 30 to 300 CFU/plate and the average was recorded.

Ice-cream preparation

Ice-cream was prepared with a domestic ice-cream maker (Il Gelataio ICK 5000, De Longhi, Italy), according to the process of Figure 1. Commercial fresh whole milk and sugars were used. The commercial base has to be used with a “warm process”, which means that it has to be mixed with the other ingredients and pasteurized. Literature (BahramParvar et al., 2012) indicates a flash treatment at 80 °C for 25 sec, followed by high pressure homogenization and maturation at 4 °C for a few hours. The process had to be adapted to laboratory facilities. Milk was pre-heated to 40-45 °C in a water bath set at 90 °C. Sugar was added and mixed with a

domestic beater (Ariete, Mixy210, 210 W). The mixture was left in the water bath until reaching a 80 °C temperature for 25 sec, rapidly cooled in water-ice bath until 10-12 °C and kept at 4 °C overnight (12 h). The final mixture was used for ice-cream making with a 30 min beating/freezing time, after which it was frozen, stored for 2 days at -18 °C and then analysed for CFU count as reported in section 2.2.

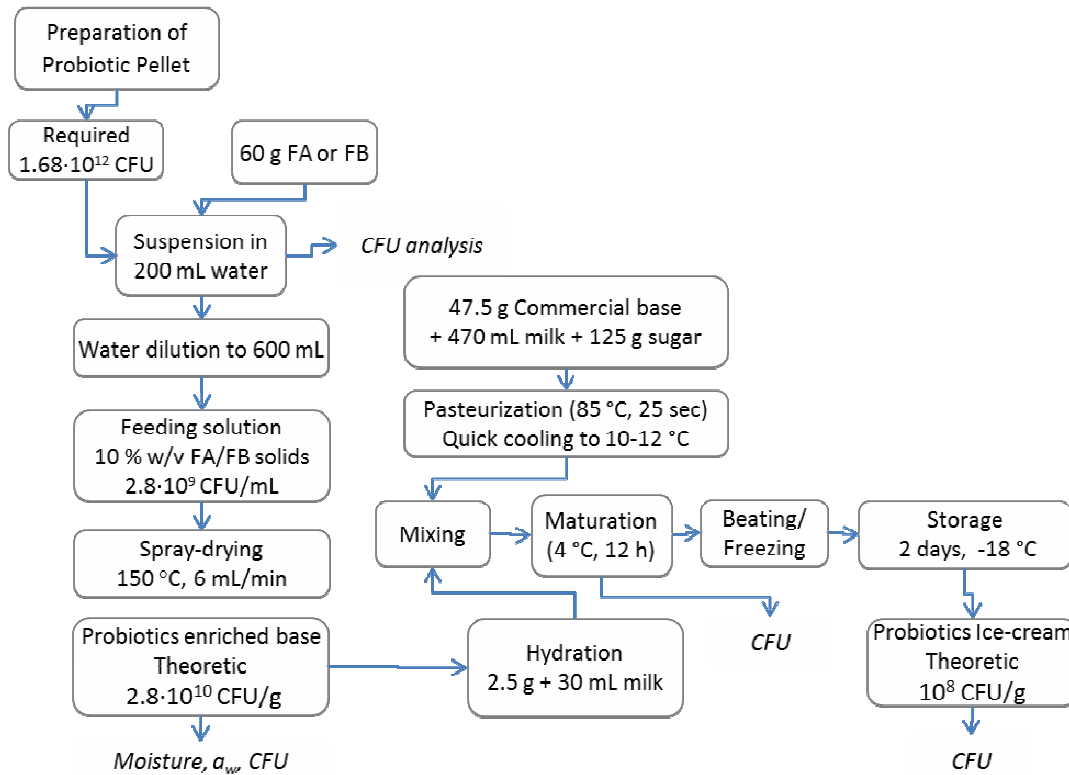


Figure 1: Process scheme for the preparation of probiotics enriched ice-cream.

Statistics

The values are reported as means \pm SD, except for a_w which was measured on one sample for each spray-drying trial set. IBM SPSS® 20.0 (SPSS, Chicago, IL, USA) software for Windows was used to perform statistical analysis of variance (ANOVA) followed by Tukey's post hoc test (for means discrimination) to assess the significance of variation among the different spray-drying trials. Variance homogeneity was confirmed according to Levene's test. All significance tests were conducted at $P \leq 0.01$.

Results and Discussion

Preparation of modified ice-cream base formulations

The idea behind this research is the development of encapsulation formulations for delivery of functional ingredients, such as probiotics, into food matrixes using materials that are already components of the final product. A commercial base for full cream ice-cream was selected for the study, since a milk-based ice-cream could represent a nutritional complete lunch substitute. Direct spray-drying of the base, even at an inlet concentration below 10 % w/v, was not possible since no powder could be recovered, probably due to the excessive content of emulsifiers and stabilizers. It was then decided to select only some of the base components taking into account the literature about probiotics encapsulation, as reported in section 1. The specific types of maltodextrins and inulin (Maltrin® 40 and Fibruline®) were suggested by the relative suppliers as the most suitable for ice-cream application. The spray-drying results are reported in Table 1.

Table 1: Results of spray-drying trials. The values followed by different superscript letters in the same column were statistically different according to ANOVA and Tukey's post-hoc test. FA: formulation with Maltrin® 40; FB: formulation with Fibruline®; FBP: FB with probiotics.

Feed volume (mL)	Formulation	Total Solids Yield (%)	Powder Dry matter (%)	a_w	T_{outlet}
100	FA	62.07 ± 4.42 ^a	96.23 ± 0.21 ^a	0.2774	65.00 ± 1.73 ^{ab}
200	FA	59.92 ± 2.43 ^{ab}	95.97 ± 0.07 ^a	0.2509	64.50 ± 1.50 ^{ab}
300	FA	60.80 ± 0.34 ^a	96.38 ± 0.54 ^a	0.2413	65.67 ± 0.58 ^a
400	FA	58.14 ± 0.46 ^{ab}	94.34 ± 0.13 ^b	0.2744	65.50 ± 1.50 ^a
100	FB	65.55 ± 5.71 ^a	96.12 ± 0.14 ^a	0.2609	62.00 ± 0.00 ^b
200	FB	60.16 ± 2.14 ^{ab}	96.22 ± 0.27 ^a	0.2482	67.50 ± 0.50 ^a
300	FB	59.15 ± 1.11 ^{ab}	96.04 ± 0.13 ^a	0.2425	65.50 ± 0.50 ^a
400	FB	59.46 ± 0.42 ^{ab}	93.56 ± 0.27 ^b	0.2952	64.50 ± 0.50 ^{ab}
600	FBP	50.89 ± 1.60 ^b	88.72 ± 0.63 ^c	0.4230	61.80 ± 1.50 ^b

Experimental data showed the possibility of obtaining spray-dried powders from both the investigated formulations. The carrier composition did not substantially influence the total solids yield, while a slight decrease with increasing feed volume was observed. This is due to a progressive fouling of the equipment atomizer, which will of course limit the operation time of an hypothetical industrial scale process. The yield values are in the range of literature results,

even though higher yields are reported for different process conditions and carrier composition (Fontes et al., 2014). Outlet temperature was almost the same in all the trials and below 70 °C. Residual moisture was not influenced by the formulation, but it was statistically lower when 400 mL were spray-dried. Water activity of all the samples was below 0.3, which is very positive for powder stability since it represents less free water available for biochemical reactions and hence longer shelf-life (Fritzen-Freire et al., 2012).

Visually the two modified bases were similar but slightly different than the original base which is more yellow and fine (Figure 2). The latter difference can be easily overcome with a milling step, while the colour depends on the composition (the original base contains full cream and concentrated milk proteins).

Both the formulations were then tested for probiotics incorporation.

Preparation of probiotics enriched formulations

The microorganism used in this study is a strain of *L. paracasei*, a species widely employed for probiotic aims in the market of the nutritional integrators. The strain was isolated by AAT from faeces of healthy child and studied for both the safety of use in humans and for its probiotic *in vitro* and *in vivo* efficiency. In particular, according to the EFSA guidance (2012), the strain was tested for antibiotics sensitivity showing to be sensible to the 8 antibiotics indicated by EFSA, which assures its safety for human consumption. Furthermore, the strain revealed to be able to survive after the transit through the gastro-intestinal barrier, and to stimulate dendritic cells (which represents an anti-inflammatory profile).



Figure 2: Original commercial ice-cream base (on the left); modified base with formulation A (in the middle); modified base with formulation B (on the right).

Interestingly, when FA was used, no powder could be collected, indicating interactions between the microbial cells and Maltrin® 40. This might be due to the type of maltodextrin, to the

microorganism species or to the specific encapsulating material composition, since other authors have succeeded in spray-drying probiotic cultures with maltodextrins. Ying et al. (2012) encapsulated *L. rhamnosus GG* using whey protein isolates, inulin, glucose and our same Maltrin® 40. Sohail et al. (2012) spray-dried *L. rhamnosus GG* in co-culture with *L. acidophilus NCFM* using sodium alginate and maltodextrin (Fieldose 10C). Even though maltodextrins have been used as a carrier material in many studies, they have a potency to penetrate the cell membrane which is largely dependent on their molecular weight (Semyonov et al., 2010).

The total solids yield in the presence of probiotics was lower (Table 1). This might have been caused by the higher volume of feed solution, and by a slightly higher inlet solid concentration due to the pellet contribution. More probably, other components of the pellet, such as cell wall polysaccharides, may have increased stickiness of the powder during the process. The most negative result was the higher moisture content of the powder, and the water activity above 0.4 which can seriously compromise powder stability and cell vitality during storage.

Compared to the required cell concentration in the feed (Figure 1), the actual measured value was slightly higher ($5.7 \cdot 10^9$ CFU/mL). Considering the actual feed load, supposing that the product lost in the plant has the same composition of the recovered product and assuming no vitality loss during the process, a theoretical value of $5.2 \cdot 10^{10}$ CFU/g in the final powder was calculated, The measured value was $9.5 \cdot 10^9$ CFU/g revealing a 82 % viability reduction during drying. The result is in agreement with literature, where Sohail et al. (2012) reported cell death ranging from 73 to 92 %. Even though spray drying is a well-established process characterized by high production rates and relatively low operational costs, the typical high working temperature can cause cell death due to simultaneous dehydration and thermal inactivation. The drying temperature of this study (150 °C) was selected based on literature works on spray-drying microencapsulation of probiotics (De Castro et al., 2012; Fritzen-Freire et al., 2012). Lower temperature have also been used, such as 140 °C by Malmo et al. (2012) for *L. reuteri*, or 120 °C by Sohail et al. (2012) for *L. rhamnosus* and *L. acidophilus*, and could be tested to improve process survival.

Ice-cream preparation

Ice-cream was prepared substituting only a 5 % of the commercial base with the modified base (formulation B enriched with probiotics). This low substitution level should reduce the influence of base modification on technological performances of final product (such as color, melting rate, overrun and melting destabilization) but, especially, it allows the addition of the probiotic ingredient after the pasteurization. Considering the dosages reported in Figure 1, the probiotic concentration in the ice-cream mixture before beating and freezing was calculated in $3.5 \cdot 10^7$ CFU/g. The viable cells enumeration of the matured mix after 3 days storage at 4 °C gave $4.3 \cdot 10^7$ CFU/ml, corresponding to $3.79 \cdot 10^7$ CFU/g (taking into account the mixture density of 1.135 g/ml). This shows that the encapsulated cells remained vital after rehydration. Analysis of the final ice-cream after 2 days storage at -18 °C, gave $3.1 \cdot 10^7$ CFU/g count corresponding to a limited additional 18 % mortality of. Stability during longer storage period has, anyway, to be further evaluated.

Conclusions

This study showed the possibility of producing spray-drying carrier materials suitable for functionalization of commercial ice-cream bases, since containing the same bases ingredients. Two formulations were developed, one with maltodextrins, widely used in the food industry and spray-drying processes, and one with inulin, a prebiotic fibre with both beneficial effects for humans and probiotics preservation. Process yield (58-66 %) and powder a_w (< 0.3) were not influenced by the formulation type. However, the maltodextrins formulation could not be used for probiotics encapsulation (null process yield), while inulin formulation gave a lower yield (51 %) and higher a_w (> 0.4). A 82 % death cell occurred during drying, but the cells survived in the ice-cream making process. The results represent an important starting point for the development of encapsulated multiple bioactive ingredients containing probiotics, prebiotics and other functional compounds such as polyphenols.

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Chapter 4 – Updating the criteria for probiotic selection and characterization for human use

Updating the criteria for probiotic selection and characterization for human use

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Manuscript in preparation

The regulatory pressure introduced in recent years in Europe led to a practical limitation of the communication of beneficial effects of probiotics to consumers and a theoretical detrimental impact on probiotic market. Almost nine years after the enter into force of the Regulation (EU) 1924/2006 on health claims made on food the market of probiotics looks still viable and dynamic since the big pool of information sorting out from the omics approach to the study of the microbiota is producing a renewed interest about the health improving potential of beneficial microbes. Legislative framework seems to be not ready to cope with the innovation associated to the development of new analytical approaches. In fact of the about 300 requests of health claims presented to EFSA concerning the food area none has been accepted, except that referred to the use of yoghurt for lactose intolerance (Papadimitriou et al. 2015). The development of new and exhaustive models, taking advantage of the newest omics technology will contribute to the understanding of the mechanism of action of probiotics and the assessment of a cause-effect relationship between the administration of probiotics and a quantifiable beneficial impact on health.

Traditional in-vitro and ex-vivo screening methods of new probiotic candidates are now coupled with omics techniques in order to speed up the selection process, to better understand mechanisms underlying their functionality and to explore the opportunity to use cell fractions instead that viable probiotics in finished products.

The widely accepted approach to the selection of probiotics includes first the assessment of the absence of pathological and virulence traits in order to assure the safe use for human consumption, and then the evaluation of the functionality of the strain in terms of prophylactic, therapeutic or as a reduction of the risk to develop a certain condition. The significance of this validation results directly proportional to the availability of measurable biomarkers associated to a certain health or disease condition.

Conventional in-vitro approaches to the selection of new probiotics

Guidelines focused on the evaluation of probiotics in food have been published already several years ago (Joint FAO/WHO Working Group, 2002) focusing on some key aspects for the characterization of bacterial strain as probiotics in food and designing a scheme of selection that represents the basis for the conventional assessment of probiotic properties. The utility of in-vitro assays relies mainly on their presumptive predictive value in order to predetermine, in

possibly reliable, fast and easy mode of action, the possible association between the probiotic candidate strain and a certain health claim, targeting a general or a specific application. Despite the efforts made by regulatory and international bodies in order to suggest a certain degree of standardization of in-vitro tests, they still show high variability in terms of reproducibility. These assays should be followed and /or partially replaced by in-vivo tests that anyway are often time-consuming, expensive and not feasible for ethical reasons.

Persistence and survival in the human gut

Probiotics are required to survive the passage through the GIT remaining in viable state with the sufficient number of cells facing the stressful conditions of the human gastrointestinal tract. Simulated gastric and pancreatic juices have been extensively used (Charteris Et al., 1998; Lavermicocca et al., 2008; Gorbach & Goldin, 1992) as well as bovine and porcine bile to check the ability of probiotics candidate to face the stressful gut environment, mainly due to the fact that these assays are easy to perform and cheap compared to other more recent approaches such as GIT simulators. Many biases have been associated to this in-vitro approach (Papadimitriou et al., 2015) since the unrealistic conditions applied during in-vitro assays that expose probiotic candidates to very harsh environmental parameters that do not mimic in efficient and realistic way the acidity of the stomach and lower intestine during food consumption.

Safety for the use in human nutrition

The relevance of some safety issues for the use of probiotics in food has been underlined by the Joint Working Group FAO/WHO in 2002 that still represents the reference guidelines for the assessment of the safety status of probiotics. The determination of the minimal inhibitory concentrations (MICs) towards some antibiotics is suggested by EFSA (EFSA Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, 2012) that also defined the microbiological cut-off values to evaluate a new probiotic candidate as susceptible or resistant to a pool of 9 antibiotics as well as the reference methodology to assess the sensitivity.

Other safety aspects include hemolytic activity, aggregation to platelets, to fibronectin and to fibrinogen, the synthesis of certain enzymes and the production of biogenic amines that are usually assessed by in-vitro tests. Nevertheless, some doubts exists about the reliability of these

assays based on the fact that the level of expression of these traits in-vivo could be significantly different from the in-vitro conditions leading to an under- or an over-estimation of some potentially worrisome features of probiotics (Papadimitriou et al., 2015).

Colonization ability

The investigation of this aspect is usually based on the ability of probiotic cells to interact with the host. Several in-vitro assays have been developed in order to evaluate the adhesion of bacteria to mucus and or epithelial cells despite the fact that this trait could have positive connotations associated to the competition with pathogens for the adherence sites as well as negative implications such as the increasing risk of translocation. Moreover, adhesion to epithelial cells, such as Caco-2, HT-29, etc, produce partially reliable results due to the low reproducibility level of the tested conditions. Other solutions have been proposed taking into consideration resected gut tissue or whole tissue models including the mucus layer but still presenting reliability bias (Vesterlund et al., 2005; Tassel and Miller, 2011). Some physical features of probiotic cells have also been taken into consideration to evaluate the ability of a strain to interact with the host such as the hydrophobicity of the cell surface whose scientific significance is considered rather limited because of the lack of specificity of this feature. Even the expression of some enzymatic activity such as GADP (glyceraldehyde-3-phosphate dehydrogenase) mediating the attachment to the mucus has been considered as screening parameter with scarce representativeness.

All of the possible adhesion assays are performed on single and purified strains and therefore the assay conditions cannot reproduce the real bacterial community structure and the interactions among its inhabitants, leading to doubts about the significance of the results and their transposition to the gut microbiome.

Antimicrobial potential

Probiotics are able to exert a competitive inhibition towards pathogens by several mechanisms including the production of metabolites with antimicrobial activity. This action has been proven to be at large spectrum against other bacteria, fungi and viruses and is based on different compounds like organic acids, bacteriocins, etc. Different in-vitro models have been developed, from the simplest ones based on diffusion in agar to more advanced systems to reveal the release of antimicrobials by the use of indicator strains. Besides the excretion of metabolites,

probiotics can also act by co-aggregating with pathogens and/or enhancing the integrity and functions of the intestinal barrier. In the first case, probiotic cells bind pathogenic cells determining a clumping effect that limits the interaction of pathogens with the surfaces of the host and facilitates the excretion of pathogens by biological fluids (saliva, feces, etc). The gut barrier function relies on tight junctions whose integrity represents a key issue to limit the translocation of bacteria, pathogens included. Probiotics have been recognized to improve the integrity of the barrier as measured by the quantification of specific enzymatic activities.

Similarly to what has been observed for the other described in-vitro models, also the evaluation of antimicrobial potential of probiotics is characterized by some biases since the laboratory conditions cannot reproduce the in-vivo situation at the level of expression of the real antimicrobial potential of the tested probiotic strain is not assured as well as for the complex interactions that characterize the microbial natural community of the GIT.

Immunomodulatory actions

This effect has been associated to the ability of probiotics to stimulate the secretion of antibodies by host cells as well as to trigger cell-mediated immune responses. These effects are mediated by dendritic cells (DCs) or M cells from the Payer's patches whose response to the presence of bacterial cells is evaluated by models based on co-culture of probiotics and immune cells and by detecting and quantifying of cytokines. These models are usually applied to the selection of probiotics with anti-inflammatory potential useful for chronic inflammatory diseases such as IBD or to be applied in the management of allergic symptoms due to their pro-inflammatory profile. Nevertheless, a strong debate exists about the modality of action of probiotics in-vivo and on how to translate in-vitro findings to the evaluation of the efficacy of probiotics in the modulation of the gut and systemic immunity.

In-vitro models of cardiovascular diseases

Probiotic candidates are usually screened for the expression of the bile salt hydrolase activity (BSH) that allows the deconjugation of bile acids and that can be evaluated qualitatively and quantitatively in relatively easy and quick ways (Cani and Van Hul, 2015; Dashkevicz and Feighner, 1989; Zheng et al., 2013; Tomaro-Duchesneau et al., 2014). These models are anyway considered by some authors largely insufficient to measure the contribution of probiotics to

reduce the risks associated to cardiovascular diseases (Papadimitriou et al., 2015), even if the beneficial action seems to be supported by scientific evidences (Ebel et al., 2014).

In-vitro anti-cancer models

Probiotics have been proven to strengthen the protection functions of the body of the host by improving metabolic and immunologic parameters. Beneficial bacteria can in fact exert antimutagenic and antigenotoxic activities as well as to degrade nitrosamines and heterocyclic amines whose accumulation in the gut could lead to potential detrimental effects. Apart from sequestering dangerous metabolites, probiotics are also recognized to release in the lumen short chain fatty acids (SCFA) with proven anticarcinogenic effects in-vivo (Burns and Rowland, 2004; Pool-Zobel, 1996; Duangjitcharoen et al., 2014; Castro et al., 2010; Faridnia et al., 2010). It is common opinion of researchers that these in-vitro tests are not sufficient to provide information about the anticancer activity of probiotics since the in-vivo validation is believed to be necessary, despite a very promising background is already available (Chong, 2014).

Other in-vitro models have been developed to evaluate reliably and fastly the probiotic profile of beneficial candidates such as measurement of B-galactosidase activity to improve the symptoms of lactose intolerance, the production of vitamins, the ability to degrade oxalate as well as to inhibit oral strains responsible for the production of volatile sulfur compounds (VSCs).

Development of innovative screening platforms

The requirement for standardized in-vitro assays to evaluate and validate probiotic effectiveness is underlined in many cases in order to draw experimental protocols with improved reproducibility and therefore decreasing the percentage of false negative and positive responders.

In-vivo models can integrate in-vitro ones in order to drive the choice of the right probiotic for a certain application.

Insect, worm and animal models, mainly rodents, dogs, monkeys and swine, have been considered despite the fact that they seem to lack predictivity for the situation in humans and therefore a certain degree of criticism should be applied when transferring speculations from animals to humans. Despite a general doubtful position emerging especially in the latest years about the conventional screening pipeline for probiotics (in-vitro assays – animal model –

clinical trial) including mice or rats models and the tendency to check directly in small number of humans the findings from in-vitro evidences, animal models will inevitably be continued to be used especially to mimic some specific diseases, induced by chemicals or in genetically predisposed animals, or infection conditions with specific pathogens. Such models are successfully applied to the study of probiotic efficacy in modulating intestinal inflammation (chemically-induced gut diseases such as DSS and TNBS, *Citrobacter rodentium*, *Clostridium difficile*, *Salmonella thiphymurium*, listeriosis), in preventing carcinogenesis, in restraining metabolic disorders, in contributing to limit the side effects of therapies for auto-immune diseases. Moreover, models of the so-called humanized mice or rats, consisting of immune-deficient animals carrying human tissue or genes, are used more to test new drugs than for probiotic validation (Papadimitriou et al., 2015).

The frontier, represented by in-vitro coupled with in-vivo test models, has been crossed by in-silico models developed in EU to take into consideration in a mathematical model the interaction between host gut, nutrients, epithelial cells and microbes of the gut community (Teusink et al., 2005; Tan and Liong, 2014) in order to simulate the high level processes taking place in the complex gut environment with predictive function regarding the interaction of probiotics with these multiple factors.

Omics approaches gave in recent years a significant contribution to the comprehension of the efficacy of probiotic towards their host, mainly quantifying by molecular methods the increased/decreased expression of certain housekeeping genes of the probiotic cell in answer to different environmental conditions. Some area on interest have been particularly investigated with special reference to those related to resistance to stressful conditions, adhesion to host tissues, modulation of the immune system, production of antimicrobial substances and nutrient compounds, degradation of prebiotics and quorum sensing.

Omics technology will surely contribute to elucidate the ongoing communication dynamics inside microbiome and between it and host tissues and the impact of an exogenous probiotic strain entering this homeostatic situation.

As reported in this manuscript, the number of available assays to validate in-vitro and in-vivo the efficacy of probiotics is huge. They are variably explanatory and not always reproducible, and in some cases indications about the efficacy of a new probiotic candidates can be retrieved only by a combination of several assays.

Nobody has defined a clear workflow to select probiotics carrying to reliable and unquestionable proof of efficacy, apart from in-vivo testing on humans by appropriately designed clinical trials. Due to the very significant costs associated to human studies, probiotics are still screened by in-vitro tests with special reference to the application of the simplest and cheapest ones to screen huge numbers of bacterial candidates and of the more expensive and appropriate ones to the validation of a more limited number of strains coming from the previous screening process.. This flow of activities is known as “funnel-like” approach that has been applied to this PhD thesis research work to perform the reasoned screening of a pool of lactobacilli and bifidobacteria newly isolated from biological samples in a relatively quick and easy way, to move then to their characterization by more complex models, developed in collaboration with research and clinical bodies, working in specific fields of human health and nutrition. Aim of the first screening process was to identify strains responding to basic criteria of reliable taxonomical allocation to a defined species as well as of assessment of the safety profile of bacterial candidates to be used in humans. Conventional models have also been applied to the selection of strains, univocally and safely identified, able to survive the passage through the GIT in viable form and therefore resistant to gastric and pancreatic juices and to bile salts, despite the recognized bias of this approach, neglecting sometimes promising probiotic candidates or carrying to the discarding of false negative survivors.

Strains evaluated as responding to the basic conventional criteria of selection were then moved to more expensive and time-consuming in-vitro and animal models in order to screen them following the efficacy for a certain predefined application on human health.

The validation of the efficacy of some strains to a certain area of human health would lead to the development of screening platforms, carrying from a large pool of probiotic candidate strains to a restricted group of probiotics specifically and univocally selected for a specific human district requiring a certain therapeutic and/or prophylactic action.

This manuscript focused on a survey of the current knowledge about models of screening and validation of new probiotics and to the description of some platforms developed as paradigm of a rationale selection process conducting from a huge amount of strains to a univocal association *one strain-one function*.

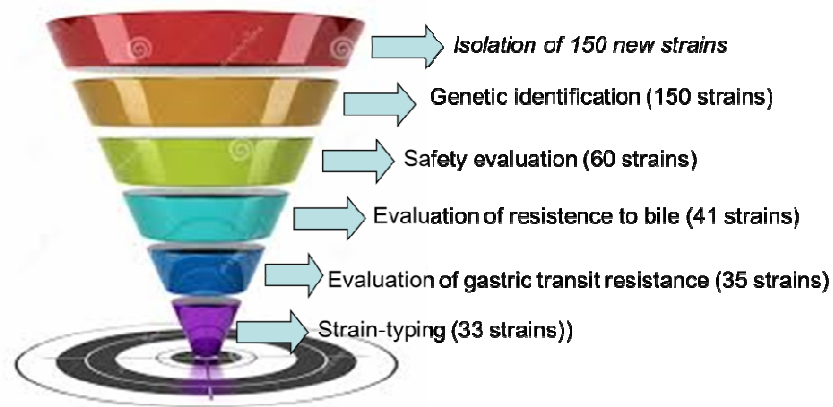


Figure 1. Scheme of a screening approach to new probiotic candidates by conventional in-vitro assays

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***Chapter 5 – Lactobacilli and Bifidobacteria
probiotics ameliorate Experimental
Autoimmune Myasthenia Gravis and
Encephalomyelitis***

Lactobacilli and Bifidobacteria probiotics ameliorate Experimental Autoimmune Myasthenia Gravis and Encephalomyelitis

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Abstract

Probiotics are viable non-pathogenic bacteria able to modify metabolism and induce immunomodulatory activity. They can modulate dendritic cell maturation, modify T helper cell balance and induce regulatory response and immunological tolerance, representing a therapeutic strategy in autoimmune diseases.

We selected newly identified live strains of probiotics, *Lactobacillus crispatus* (LMG P-9437) and *Bifidobacterium breve* (LMG S-28195), and tested their properties in immune-modulating dendritic cells and inhibiting auto-reactive effector T cells, via TGF β production. Innovative combinations of probiotics were tested in vivo in the animal models of Myasthenia Gravis and Multiple Sclerosis, showing immune-modulatory effects both locally in Peyer's Patches, and systemically, inhibiting auto-immune inflammation in the target organs and ameliorating clinical symptoms.

Here we showed preclinical data, highlighting the therapeutic efficacy of the newly selected probiotic strains, as potential translational approach for human autoimmune diseases.

Introduction

Probiotics are live non-pathogenic bacteria known to have beneficial effects on the host organism mostly associated with modulation of immune responses (Perdigon,G. 1995). The gastrointestinal tract is the primary site of interactions between the host and the microbiota. How bacterial colonization of the gut might influence the development and functions of the immune system has become a major focus of interest (Surana,N.K. 2014; Erickson,K.L. 2000). The microbiota has a positive impact on immune regulatory functions of the gut, and interest has emerged to address the potential role of probiotics in the induction (or restoration) of regulatory-type immune responses; moreover, intestinal colonization with commensal bacteria is critical for the establishment of oral tolerance (Weiner,H.L. 1997). Recent studies suggest that an impaired intestinal barrier function might cause an imbalance between Th1/Th2 responses, thus triggering autoimmune processes (Fasano 2005; Fasano,A. 2012). Auto-reactive Tcells are normally present in all individuals, but mechanisms for peripheral self-tolerance exist to control potentially pathogenic Tcells (Piccirillo 2004). Peripheral tolerance is a continuous process, occurring when mature lymphocytes, escaped from negative selection during ontogeny, encounter self-antigens in secondary lymphoid organs. The mechanisms that control peripheral tolerance include ignorance, deletion by apoptosis, the induction of anergy, and active suppression, which is mainly realized by specific cell subsets of regulatory Tcells (Treg) (Bacchetta 2005).

In this context, the immune-modulatory properties of probiotics have raised great interest. Reviews of several clinical studies support that probiotics may represent a capable preventive and therapeutic strategy for allergic and chronic inflammatory diseases (Boyle,R.J. 2006). Even if it is reported that they can mediate a control of the balance between pro- and anti-inflammatory cytokines (Di Giacinto,C. 2005; Amati,L. 2010) the mechanisms underlying these beneficial effects are not completely understood but have been associated with immune-modulatory properties of specific probiotic strains (Erickson,K.L. 2000). Among the complex human microbiota, lactobacilli, more predominant in the gastric region and upper gastrointestinal tract (GIT) and bifidobacteria, mostly present in the lower intestinal tract, showed therapeutic effects in rheumatoid arthritis (So,J.S. 2008; So,J.S. 2008), inflammatory bowel disease (Kim,N. 2007; Castagliuolo 2005) and atopic dermatitis (Viljanen,M. 2005). These therapeutic effects are achieved through regulation of cytokine expression and modulation of

DC function associated with an enhanced generation of regulatory DCs expressing high levels of IL10, TGF β and COX2, and an enrichment of CD4⁺CD25⁺ Treg cells in the inflamed regions (Baat 2004; Braat 2004; Kwon 2010; Christensen 2002). Single strains (Lavasani,S. 2010) or combinations of probiotics (Chae,C.S. 2012) have emerged as promising candidates for treatment of inflammatory and autoimmune disorders. Amongst these, Myasthenia Gravis (MG) and Multiple Sclerosis (MS), known to be characterized by an imbalance between T effector/Tregulatory cells (Thirupathi 2012) are of interest due to their highly invalidating impact on everyday life. MG is a systemic autoimmune disease leading to muscle weakness and fatigability, where the nicotinic acetylcholine receptor (AChR) is the major auto-antigen involved at the neuromuscular junction (Conti-Fine 2006). Experimental autoimmune myasthenia gravis (EAMG) is a well-established animal model for MG and has been extensively studied to elucidate the pathogenic mechanism (Ubiali 2008; Marolda 2013). MS is a chronic, T cell-dependent, inflammatory autoimmune disease of the central nervous system (CNS), whose animal models, i.e. experimental autoimmune encephalomyelitis (EAE) shares a number of clinical, genetic and immunological features with the human disease (Steinman,L. 1999), and can be used as prototype-model for new therapies.

In the present work we identified and investigated strains of lactobacilli and bifidobacteria, studying their immune-regulating effects both in vitro and in vivo, combining their action to commercially available strains. Treatment of DCs with probiotic combinations led to strong immune modulation, able to inhibit auto-reactive effector T cell activation. Oral administration provided regulatory effects with an up-regulation of TGF β transcript and protein on local gut lymphoid tissue, showing therapeutic properties, thus ameliorating clinical symptoms in both EAMG and EAE models.

Results

Combinations of novel probiotics efficiently immune-modulate gut lymphoid tissue.

Different strains of live probiotics were tested among a selection of newly identified strains of lactobacillus and bifidobacteria. We focused on specific strains of bifidobacteria, known to produce anti-oxidant and anti-inflammatory molecules, such as conjugated Linoleic Acid (CLA). In particular, *Bifidobacterium breve* (LMG S-28195; BB), a new identified strain, was able to produce this molecule in higher percentages (42.2% of converted CLA) as compared to the commercially available strain *Bifidobacterium animalis* subsp. *Lactis*, BB12® (BL; 27.1% of converted CLA) or to species of lactobacilli, among which the probiotic strain of *Lactobacillus crispatus* (LMG P-9437; LC) and a commercially available strain of *Lactobacillus rhamnosus* (ATCC 530103; LR). LC was chosen for its anti-inflammatory properties as previously reported (Voltan 2008; Voltan 2007; Castagliuolo 2005). To strengthen the genus-specific effect of either lactobacilli or bifidobacteria, we decided to use probiotic combinations of LC+LR and BB+BL. In order to test the in vivo gut colonization after treatment with LC+LR or BB+BL, rat fecal samples were collected at different time points (Suppl. Figure 1A). Following the exogenous administration of probiotics to rats, lactobacilli were found to persist and were retrieved from feces during the administration period as well as during the wash-out (Suppl. Figure 1A). Conversely, bifidobacteria increased in feces following 2-wk administration while in the subsequent time points total bifidobacteria returned to lower levels (Suppl. Figure 1A). Since bifidobacteria are not usually present in significant amount in rodent gut, while lactobacilli are natural inhabitants of the GIT, the observed persistence dynamic is in line with previously published studies (Elli 2006; Bartosch 2005), showing that it is difficult to increase the population of lactobacilli and bifidobacteria in healthy subjects with initial high fecal counts of these microbial groups.

To study the local immune-regulatory effects of probiotic combinations, the gut-associated lymphoid tissue of mesenteric lymph nodes (mesLNs) and Peyer's Patches (PPs) was analyzed, together with the systemic compartment of peripheral blood leukocytes (PBL). The percentage of CD4⁺CD25⁺ Treg increased following 2-wk treatment with bifidobacteria in PBL, PPs and mesLNs (Suppl. Figure 1B). Alongside, the supernatant of ex-vivo cultured mesLN cells derived from BB+BL-treated rats showed a significant increase in TGFβ protein level compared to mesLNs from vehicle-treated animals (Suppl. Figure 1C). Moreover, we analyzed the gene

expression levels of immune-regulatory markers in mesLNs and PPs, after 4-wk treatment showing higher expression of FoxP3, CTLA4, CCR7 and TGF β , only in PPs (Suppl. Figure 1D). Interestingly, bifidobacteria induced a persisting immune-regulation in PPs even after wash out (T4), despite the observed slight decrease in bacteria counts.

Bifidobacteria ameliorate Experimental Autoimmune Myasthenia Gravis.

We studied the immune-regulatory effect of the newly identified probiotic strains in the EAMG model. Rats immunized with *Torpedo californica* Acetylcholine receptor (TACHR) were treated with combinations of probiotic strains, either lactobacilli or bifidobacteria, following a combined preventive-therapeutic schedule (Figure 1A). EAMG clinical manifestations (clinical score and body weight, Figure 1B-C respectively) were significantly ameliorated by both LC+LR and BB+BL administration with the latter being more efficient, already at an earlier disease stage (7 wks p.i.). Interestingly, at the level of primary responses in draining lymph nodes (drLNs), BB+BL treatment was able to partially attenuate TACHR specific Tcell proliferation (Figure 1D). Nevertheless, the systemic effect of probiotics was characterized by a significant decrease in sera anti-AChR Ab titers in BB+BL-treated (15.18 pmol/ml \pm 4.55) but not in LC+LR-treated (23.79 pmol/ml \pm 7.23) animals, compared with vehicle (43.94 pmol/ml \pm 6.16) (Figure 1E). Alongside, a clear ameliorating effect of BB+BL combination was observed locally, at the level of EAMG target tissue. Indeed, microscopic evaluation using fluorescently-labeled Bungarotoxin (BTX) demonstrated increased number of AChR clusters and AChR mean fluorescence intensity in gastrocnemius muscle of BB+BL-treated rats (Figure 2A-B). Moreover, quantitative RIA analysis demonstrated that BB+BL-treated animals showed a muscle AChR content (0.09 pmol/g \pm 0.01) similar to healthy control animals (0.091 pmol/g \pm 0.006), much higher compared to both vehicle (0.05 pmol/g \pm 0.006) and LC+LR-treated rats (0.06 pmol/g \pm 0.006; Figure 2C). Further, to evaluate the immune-regulatory effect of probiotics in EAMG, we quantified the expression levels of selected immune-related mRNA transcripts in the myasthenogenic thymic tissue (Cordiglieri 2014) and in immune lymphoid organs, PPs and spleen (Figure 2D-E). Indeed, both lactobacilli and bifidobacteria reduced the expression of inflammatory mediators, such as IFN γ and IL6, while modulating regulatory markers. In particular, bifidobacteria treatment induced an increase of TGF β in thymus and PPs (Figure 2D).

Probiotic-induced amelioration of Experimental Autoimmune Encephalomyelitis.

Since imbalance between immune-regulatory and auto-aggressive effector T cells is supposed to be a key feature in autoimmune diseases, we decided to corroborate the efficacy of probiotics in another autoimmune animal model, EAE of the Lewis rat, in which effector T cells play a major role. Animals were daily fed with probiotic combinations (LC+LR; BB+BL) according to a prophylactic schedule, prior to EAE induction (Figure 3A), then oral administration continued until disease peak, providing a preventive treatment. EAE clinical manifestations (Figure 3B) were significantly ameliorated by both LC+LR and BB+BL administration, being BB+BL treatment slightly more efficient, compared to vehicle-treated animals. The reduced clinical symptoms were accompanied by dampened primary immune responses in drLNs (Figure 3C), suggesting ongoing immune-regulation induced by probiotic treatment, in the specific district of auto-aggressive effector T cell formation. Indeed, Th1/Th17 inflammatory markers were significantly down-regulated in the spinal cord of treated rats (Figure 3D), whilst immune-regulatory genes were up-regulated locally at the level of PPs (Figure 3E). Alongside, both CNS immune cell infiltration and astrocytosis were significantly reduced following probiotic treatment (Figure 3F-G), which also prevented EAE-induced demyelination in the spinal cord (Figure 3H-I). To further confirm the effect of prophylactic-preventive probiotic treatment in blocking CNS immune infiltration during EAE, we performed transfer EAE experiments, via injection of GFP⁺ encephalitogenic myelin basic protein (MBP)-specific T cell blasts (Figure 4A) and evaluated T cell migration and accumulation in lymphoid tissues and CNS. Indeed, both lactobacilli and bifidobacteria reduced GFP⁺ T cell infiltration in lumbar spinal cord, whilst retaining encephalitogenic blasts in peripheral immune organs (Figure 4B-D).

Probiotic-induced TGFβ production by DCs inhibits effector T cell activation and immunological synapse formation.

To address the mechanism through which the combinations of newly identified probiotic strains exert their functions, we tested in vitro the effects of single and combined probiotics on DCs, derived from rat bone-marrow myeloid precursors (Figure 5) and human peripheral blood mononuclear cells (PBMCs) (Suppl. Figure 2). Probiotic treatment induced DC maturation, as visualized by the enrichment in activation surface marker expression: CD11c, CD80, MHCII and CD103, for rat DCs (Figure 5A,B), CD80, CD86, MHCII and CD83 for human DCs (Suppl. Figure 2A). Importantly, probiotic combination-induced DC maturation was accompanied by an

immune-modulatory phenotype characterized by higher expression levels of TGF β , CCR7 and IL6 (Figure 5C; Suppl. Figure 2B), compared to control samples. Interestingly, this immune-modulating phenotype was observed also on DCs treated with combination of heat-inactivated probiotic strains (Figure 5D). DCs incubated with heat-inactivated probiotics, especially with BB+BL, were characterized by high expression levels of TGF β and CCR7, accompanied by increased Aldh1a and reduced TLR4 (Figure 5D) and high TGF β protein levels (Figure 5E). These data suggest that proteoglycan residues present on the bacteria shell may modulate DCs, even when probiotic agents are not alive (Taverniti, V. 2011).

To address the immune-modulatory effects of probiotics on DCs, we used an in vitro co-culture set-up, which mimics the in vivo environment leading to antigen-specific T cell activation. Indeed, we studied co-cultures of antigen (Ag)-loaded DCs in presence of Th1/Th17 effector T cells (Teff) specific for either MBP or AChR (using the rat immunogenic epitope of the α -subunit of AChR; R97-116), similarly to previously published experiments (Cordiglieri 2014). Specifically, we analyzed the cellular interactions between MBP- and R97-116- specific Teff and Ag (MBP or R97-116)-loaded DCs, treated or not with heat-inactivated probiotic combinations of LC+LR or BB+BL (Figure 6A). When co-cultured with Ag-DCs, Teff showed a random movement (Miller 2004) (Figure 6B; Suppl. Video 1), and then acquired a stationary phenotype when encountering the proper MBP-MHC complex, showing a relatively stable low Vmax (4 μ m/min), and remaining fixed to anchor points (Cordiglieri 2014; Kawakami 2005). After 60 minutes co-culture, 80% of Teff showed a stationary phenotype with a minimum meandering index and Teff/Ag-DC contacts were long lasting, visually resembling immunological synapse formation. When DCs were previously treated with lactobacilli (LC+LR/DCs; Figure 6C, Suppl. Video 2) or bifidobacteria combination (BB+BL/DCs; Figure 6D, Suppl. Video 3), increased Teff Vmax were observed at 60-min co-culture, and only 30% of Teff presented a stationary phenotype. These phenomena were very similar to what was observed using DCs, which had been treated with the immune-modulating cytokine TGF β (Figure 6E, Suppl. Video 4), where we observed only 40% of stationary cells between 30 and 60 minutes of co-culture (see Suppl. Figure 3 for statistical comparisons), suggesting that probiotics treatment on DCs leads to a TGF β -like immune-regulatory milieu. Indeed, probiotic modulation of DCs promoted a marked TGF β increase in the co-culture, accompanied by a reduced inflammatory milieu which inhibited Th1/Th17 cytokine expression (IFN γ , IL17, IL2, IL2R α), as quantified by qPCR after 12h co-culture (Figure 7A). Importantly, the up-regulation of TGF β mRNA level was accompanied by a

significant increase in TGF β protein level in the co-cultures where DCs had been previously incubated with bifidobacteria (Figure 7B).

Discussion

In recent years, probiotic bacteria have been suggested to provide health benefits (Belkaid, Y. 2014). In particular, the use of specific strains as safe supplements for human consumption has been approved to enhance the gut bacterial composition. Probiotics are known for their multiple actions, including regulation of intestinal microbial homeostasis, maintenance of the gastrointestinal barrier function, interference with pathogen colonization ability, and modulation of local and systemic immune responses (Boirivant, M. 2007; Guglielmetti, S. 2011). In this context, given the intimate interplay between gut microbiota and the host immune system, it is not surprising that some probiota members have been linked to immune-modulation (Haller, D. 2010). Indeed, only recently, a putative role for probiotics has been investigated in animal models of different autoimmune diseases such as rheumatoid arthritis (Wu, H.J. 2010), EAE (Lavasani 2010), EAMG (Chae 2012), and type 1 diabetes (Markle, J.G. 2013). In particular, both single strains and probiotic combinations have been proposed as therapeutic approaches for amelioration of ongoing diseases (Lavasani 2010; Chae 2012),.

Here we focused on EAMG and EAE models and elucidated the immune-modulatory effect of probiotic agents, including two newly identified strains: *Lactobacillus crispatus* (LMG P-9437) and *Bifidobacterium breve* (LMG S-28195). We investigated the effect of specific combinations of lactobacilli and bifidobacteria, in order to enhance the genus-specific immune-modulatory action, highlighted by the in vitro data (Figure 5), where a genus-specific synergistic effect is observed. In the EAMG model, which is characterized by a primary IgM-mediated phase followed by a chronic IgG-induced disease (Baggi 2012), we decided to employ a probiotic administration schedule comprising both a preventive and a therapeutic treatment. Previous studies showed that prophylactic-preventive, but not prophylactic-therapeutic administration of commercial probiotic combination resulted in reduced clinical symptoms (Chae 2012). Differently, our approach covered both the initial and the progressive phases of the disease and resulted in its significant amelioration when the combination of bifidobacteria was administered (Figure 1). The clinical data were corroborated by analyses of neuromuscular junction morphology, muscle AChR content and anti-AChR Ab titer (Figure 2), showing

significant bifidobacteria effect. In contrast, we observed that treatment with lactobacilli was not comparably efficient (Figure 1-2), probably due to their differential pattern of gut colonization and survival and their local effect on gut-associated lymphoid tissue (Suppl. Figure 1). Indeed, bifidobacteria are known to preferentially inhabitate the lower intestinal tract (Elli 2006; Bartosch 2005), where they are easily in contact with immune cells, thus exploiting their immune-modulating function. In fact, bifidobacteria induced production of TGF β in mesLNs and up-regulation of several immune-regulatory genes in PPs (Suppl. Figure 1).

The second autoimmune disease model, in which we decided to test the efficacy of the newly identified probiotic combinations, is a monophasic, acute Tcell-mediated disease, different from the chronic progressive murine EAE previously published (Lavasani 2010). This model is particularly useful in evaluating the effects of novel therapeutic approaches on Tcell activation (Flugel, A. 2001; Cordiglieri 2010). Moreover, due to the short duration of the disease, the prophylactic-preventive administration timely coordinate bacterial colonization with disease onset (Figure 3; Suppl. Figure 1), leading to a decrease in specific effector Tcell responses, and consequently diminished clinical symptoms following both probiotic combination treatments. In such model, we did not observe statistically significant differences between lactobacilli and bifidobacteria (Figure 3). The efficacy of probiotic treatment led to diminished immune cell infiltrates in the spinal cord, whilst retaining leukocytes in immune peripheral organs (Figure 4), and reduced demyelination, astrogliosis, and neurodegeneration. In order to elucidate the mechanisms exploited by the probiotic agents, we took advantage on in vitro set ups, thus allowing dissection of the immune-modulatory effects. The four employed strains, even after heat inactivation, showed DCs modulation towards a tolerant phenotype, characterized by high levels of TGF β , thus implying a major role for bacterial shell proteoglycan residues (Figure 5; Suppl. Figure 2) (Taverniti, V. 2011). Indeed, the treatment with heat-inactivated probiotic combinations on DCs, dampened immunological synapse formation with effector Tcells, in an in vitro co-culture model (Cordiglieri 2014), resulting in diminished effector Tcell activation, Th1/Th17 inflammatory mediator production, whilst increasing TGF β (Figure 6-7; Suppl. Figure 3; Suppl. Videos 1-4).

Thus, we have shown that probiotic agents, lactobacilli and bifidobacteria, may induce a TGF β -mediated immune-modulation on DCs in vitro. Translated in the in vivo context, at the level of gut-associated lymphoid tissues (mesLNs and PPs), DCs may be prompted by probiotics to induce Treg. In experimental models involving autoimmune reactions, an increased amount of

circulating Treg may actively suppress effector Tcell functions, thus inhibiting the ongoing autoimmune inflammation.

Taken together, these preclinical data could represent a promising novel therapeutic approach in autoimmune diseases. Importantly, differently from other treatments, probiotics are safe food supplements, completely feasible with everyday life, thus implying a very good compliance for the patients.

Materials and Methods

Probiotic strain cultures

Strains of lactobacilli were grown in De Man, Rogosa & Sharp (MRS) medium (Difco, US) broth at 37° C in microaerophilic conditions for 18 h, while bifidobacteria were cultured in the same medium added with 0,05% cysteine at 37°C by anaerobic incubation for 24-48 h. Probiotic strains: *Lactobacillus crispatus* (LMG P-9437; LC), *Lactobacillus rhamnosus* (ATCC 53103; LR), *Bifidobacterium breve* (LMG S-28195; BB), *Bifidobacterium animalis* subsp. *Lactis*, BB12® (BL; from CHR Hansen, Denmark).

Strains used are considered safe, particularly *L. crispatus* LMG P-9437 and *B. breve* LMG S-28195 had already been previously isolated and characterized for their susceptibility to antibiotics and the relative data were confirmed during the thesis. *L. rhamnosus* ATCC 53103 has not antibiotic resistances while *B. animalis* subsp. *Lactis*, BB12 shows a resistance to tetracycline due to the presence of the *tetW* gene but this resistance is not transmissible so the strain does not present safety problems. More precisely, upstream of *tetW* is a transposase gene, which is cotranscribed in tandem. Transposases are involved in the horizontal gene transfer of genetic elements among bacteria, but to date there is no evidence that *tetW* in *B. animalis* subsp. *lactis* is transmissible.

Preparation of probiotic cultures administered to animals

Lactobacilli and bifidobacteria were grown in MRS broth as previously stated. At the end of the incubation period, cells were harvested by centrifugation at 8000 rpm for 5 minutes, washed twice with sterile distilled saline and finally resuspended in a solution of 20% glucose + 10% glycerol to obtain a final concentration of 10⁹ colony-forming units (CFU) per 100 µl of bacterial suspension. Concentrated cultures was then divided in single doses and aliquotes were stored at -80°C until administration to animals. Enumeration of viable bacterial cells was performed on agarized selective media (MRS for lactobacilli and Transoligosaccharide propionate agar medium TOS added with 50 µg/ml mupirocin for bifidobacteria) (Merck Millipore, US) by decimal counts just after preparation as well as along the storage in frozen conditions in order to ascertain their stability over time.

Fecal samples collection and analysis

Fecal samples were collected immediately before the first administration (T0), after every treatment week (T1, T2, T3) and after 1-week wash-out, following treatment termination (T4). Samples were immediately stored at -20°C and processed within 24 h. Fresh fecal samples were decimally diluted into sterile saline solution and plated onto selective media in order to enumerate total lactobacilli and bifidobacteria, as described above. Plates were anaerobically incubated at 37° C for 72 h. The number of colonies for two parallel plates was counted from a dilution yielding 30 to 300 CFU/plate and the average was recorded.

Animals and experimental models

Animals were purchased from Charles River Breeding Laboratories (Calco, Italy) and kept at the animal facility of the Institute. A total of 100 animals were used for all experiments and sacrificed after deep anesthesia via carbon dioxide; for immunization and treatments, animals were anesthetized with 2% isoflurane (60:40 N₂O:O₂, flow rate 0.8 L/min). EAMG and EAE were induced in female Lewis rats, aged 6-8 wks, by immunization in the hind limbs s.c., with 50 µg of TACHR (extracted from Torpedo electric organ; Aquatic Research Consultants) or 200 µg of MBP (Sigma) emulsified in CFA in a total volume of 200 µl/rat.

EAMG and EAE clinical evaluation

Evaluation of disease manifestations in EAMG rats was performed by testing muscular weakness as reported (Ubiali 2008; Marolda 2013). Clinical scoring was assessed after exercise for 30 seconds, using the grip strength test. Disease severity was graded as follows: grade 0, normal strength and no abnormalities; grade 1, mildly decreased activity and weak grip or cry; grade 2, clinical signs present before exercise (tremor, head down, hunched posture, weak grip); grade 3, severe clinical signs at rest, no grip, moribund; and grade 4, dead. Each animal was weighed and scored at the beginning of each experiment and twice weekly until the end of the experiment; EAMG was confirmed by Prostigmine test (i.p. injection). Evaluation of disease manifestations in EAE rats was performed as reported (Cordiglieri 2010). Animals were assessed daily for neurologic signs of EAE according to the following five-point scale: 0, healthy; 1, tail weakness or paralysis; 2, paraparesis (incomplete paralysis of one or two hind limbs/plegia of one hind limb); 3, paraplegia extending to the thoracic level; 4, forelimb weakness or paralysis with hind limbs paraparesis or paraplegia; and 5, moribund or dead animal.

Treatment protocols

For in vivo gut colonization, oral administrations (2.5×10^9 CFU/ml; 3wks; 5d/wk) with lactobacilli (LC+LR) and bifidobacteria combinations (BB+BL) have been performed in healthy female Lewis rats (8 wk-old, n=20). Fecal samples for biological analysis were collected at different time points: before treatment (T0), after 1, 2 or 3 wks of treatment (T1, T2, T3) and 1 wk after removal of probiotic treatment (T4). Animals were sacrificed at T2 and T4 time point. For EAMG model (6 wk-old, 2 independent experiments, total 15 rats/treatment), oral probiotic administration started from the disease induction for 2 wks (preventive treatment) and from 4 wk post immunization (p.i.) (therapeutic protocol) with strain combinations (LC+LR and BB+BL). EAMG animals were sacrificed at 10 wks p.i.. For EAE model, oral probiotic administration started one wk before immunization (prophylactic treatment) until 14 days after disease induction (preventive protocol) with LC+LR and BB+BL. EAE animals (8 wk-old, 3 independent experiments, total 18 rats/treatment) were sacrificed at the disease peak (d 21 p.i.). For EAE transfer, after prophylactic and preventive treatments, MBP-specific GFP⁺ T cells were injected at d 14 and rats were sacrificed at d 18 to perform infiltration analysis.

Immunobiological analysis

LNs were isolated from EAMG and EAE rats and processed into single cell suspensions. 2×10^5 cells/well were cultured with 0.25 μ g/ml TChR or 5-10 μ g/ml MBP (specific Ag for EAMG and EAE, respectively) and 2 μ g/ml Concanavalin A (ConA; Sigma; positive control) in 96-well flat plates in 200 μ l RPMI medium (Euroclone) supplemented with 10% FCS, 1% Na-pyruvate, 1% non-essential aa, 1% L-glutamine, 1% penicillin-streptomycin (Euroclone), 50 μ M 2-mercaptoethanol (Sigma), plus 1% normal rat serum. After 72h of incubation at 37°C with 5% CO₂, cultures were pulsed with 0.5 μ Ci [³H]-thymidine per well for 18h, and proliferation was measured from quadruplicate cultures on a beta counter (PerkinElmer).

Muscle AChR content

AChR content in muscles was assayed as previously described (Baggi 2004). Briefly, AChR was solubilized from muscle membranes overnight at 4°C in a buffer of Tris-HCl (pH 7.5), NaCl, PMSF, EDTA, plus 2% Triton X-100, after a centrifugation at 17000 \times g for 1h. Solutions containing solubilized AChR were clarified by centrifugation at 100,000 \times g for 30min. AChR crude extracts (100 μ l, duplicates) were incubated with [¹²⁵I]- α BTX (PerkinElmer) 4h at room

temperature, transferred on DE-81 DEAE disks (Whatman International) and washed with Tris-HCl buffer 0.5% Triton X-100. Radioactivity was determined by gamma counting. The aspecific binding was subtracted from each sample by parallel tubes pre-incubated with cold α BTX (Sigma). The results were expressed as picomole of α BTX binding sites per gram of muscle.

Anti-AChR Ab titer

Anti-rat AChR Abs were assayed in individual sera by RIA (Baggi 2004). Rat AChR was extracted from rat muscle and labeled with 2 nM [125 I]- α BTX. Sera were incubated over-night with [125 I]- α BTX labeled rat AChR (0.5 pmol). Ab-AChR complexes were precipitated by adding an excess of rabbit anti-rat IgG (Sigma). Pellet were washed twice with cold PBS plus 0.5% Triton X-100 (Carlo Erba) and [125 I]- α BTX labeling was evaluated via a γ -counter (PerkinElmer). Serum samples incubated with rat AChR pre-incubated in excess of cold α BTX (Life Technologies; aspecific binding) were subtracted from test samples. The anti-AChR Ab titers were expressed as picomole of [125 I]- α BTX binding sites precipitated per milliliter of serum.

Histological analysis

Ten, fifteen or twenty- μ m thick serial sections from 4% PFA-fixed and cryo-protected frozen spinal cord, spleen, gastrocnemius muscle were cut in transversal slices, using a Leica CM1950 cryostat. For AChR fluorescence labeling α BTX-Tetramethylrhodamine (Life Technologies) was employed. Images were captured via fluorescence-confocal laser-scanning microscopy (EZ-C1 scan-head equipped with Eclipse TE2000-E microscope; Nikon). Maximum projections of 15- μ m thick sections were acquired using a 20x (NA 0.85) objective and used for evaluation of AChR cluster numbers and mean fluorescence intensity (MFI) (Cole,R.N. 2008). All image settings were determined at the beginning of the imaging session and were not changed. All sections were collected in the same session permitting comparison of fluorescence intensity as a measure of relative AChR density. To avoid biased sampling, we examined for each section every adjacent field, collecting images of all fluorescently labeled AChR cluster. Single z-scan acquired using a 60x (NA 1.40) objective were employed for morphological characterization of neuromuscular junction. Histological myelin staining, performed on 20 μ m spinal cord slices using Black Gold II (BGII, Histo-Chem Inc. Jefferson), and H&E staining were performed on 6 slices per animal (n=4 for each treatment group) and captured with Aperio digital scanner equipped with 20x objective (ScanScope, Aperio Technologies). Single and double

immunofluorescence staining were performed on 6 serially cut 10- μ m thick spinal cord slices per animal (n=4 for each treatment group) with primary Abs specific for: CD3 (eBioscience), β Tubulin (Covance), GFAP (Dako), MBP (Chemicon), Desmin (Thermo-Fisher); non-immune IgG (Sigma) staining was used as isotype control. Secondary labeling was performed with Alexa Fluor 488- or 546-conjugated goat anti-mouse and anti-rabbit IgGs, and DAPI (all from Life Technologies) staining. Images were captured via fluorescence-confocal laser-scanning microscopy (EZ-C1 scan-head equipped with Eclipse TE2000-E microscope; Nikon). Maximum projections of 6- μ m thick z-stacks were acquired using a 60x (NA 1.40) objective and used for evaluation of CD3, MBP, GFAP and β Tubulin MFI values, as elsewhere reported (Colombo,E. 2012). Image analysis was performed with Image J (Version 1.43u) and FIJI (Schindelin,J. 2012): color density (for BGII), MFI and single/double-positive cells were measured on at least 3 adjacent field areas per section.

cDNA synthesis and qPCR

Total RNA was extracted using Trizol reagent, cDNA was synthesized from RNA using random hexamers, and reverse transcriptase. Real-time quantitative PCR for IFN γ , CCR7, CTLA4, FoxP3, TGF β , IL2, IL2 α , IL6, IL17, TNF α , TLR4, Aldh1a2, GAPDH and β Actin was performed using Assay-on Demand Gene Expression Products. β Actin and GAPDH were used as housekeeping endogenous genes. mRNA levels of target genes were expressed as relative values ($2^{-\Delta Ct} \times 100$) normalized towards the chosen housekeeping genes, in which ΔCt represents the difference between Ct of the target gene and Ct of the housekeeping gene. Real-time PCR reactions were performed in duplicates using an ABI Prism 7500 FAST Real-Time PCR System (all reagents and molecular probes were from Life Technologies).

Rat and human DC cultures

Single cell suspensions of myeloid precursor cells (MPCs) were derived from bone marrows of naïve Lewis rats and cultured in complete RPMI medium, containing 1% Na-pyruvate, 1% non-essential aa, 1% L-glutamine, 1% penicillin-streptomycin, 50 μ M 2-mercaptoethanol, 10% FCS, in presence of GM-CSF and IL4 (both 20 ng/ml; Peprotech) for 10 days to differentiate into immature DCs (Marolda 2013). DCs were then seeded either in live imaging glass-inserted plates (3×10^5 DCs/dish) or 24-well culture plates (1×10^5 wt DCs/well; 2.5×10^5 human DCs/well) and treated with viable or heat-inactivated (5min at 90°C) probiotics, for 4-8h in a ratio 1:100

($1-3 \times 10^7$ CFU/ sample), according to the specific assay, as specified in the respective figure legend. DCs were derived from healthy PBMCs as reported (Nava 2012), by culturing cells in complete RPMI medium in presence of GM-CSF and IL4 for 7 days.

FACS analysis

Rat and human DCs were cultured in 24-well plates (1×10^5 DCs/well) in presence of 1×10^6 CFU/well of single probiotic strains or probiotic combinations and incubated for 4h. DCs were then extensively washed and collected for cytofluorimetric analysis using specific mouse anti-human and mouse anti-rat FITC-, PE- and PerCP-labeled Abs against specific DC surface markers (MHCII, CD80, CD86, CD103, CD11c, CD83; all from eBioscience). PBL purified via percol gradient (Lymphoprep, Nycomed), together with PPs and mesLN cell suspensions obtained from 2-wk treated rats with probiotics or vehicle, were stained for FITC- and PE- labeled regulatory Tcell markers (CD4, CD25, both from eBioscience). As negative control, FITC-, PE- and PerCP-labeled mouse IgGs were used. Samples were acquired using a FACS vantage (BD) or a MACSquant (Miltenyi) and gated for Forward Scatter (FSC) and Side Scatter (SSC) parameters; 7-AAD marker (BD) was employed to visualize dead cells. Numbers of living and dead cells were counted relatively to a known amount of unlabeled beads appropriately gated (BD).

R97-116 and MBP specific effector Tcell lines

EGFP^{+/+} transgenic Lewis rats were immunized with 200 μ g of R97-116 peptide (CASLO lab) or 200 μ g of MBP in CFA as previously described (Cordiglieri 2010; Cordiglieri 2014). At d 10 p.i., drLN cell suspensions were cultured in complete RPMI medium, containing 2% rat serum and stimulated with R97-116 or MBP (5 μ g/ml). Tcells were maintained and expanded as reported (Flugel, A. 2001).

Live-imaging assay

Time-lapse video microscopy was performed using a live-imaging Nikon set-up equipped with temperature/CO₂ control unit (OKOlab). Naïve or TGF β (10 ng/ml) or heat-inactivated (5min at 95°C) probiotic-treated (1×10^7 CFU/sample) wt DCs (3×10^5 /imaging dish) and R97-116- or MBP-specific GFP⁺ Teff (1×10^6 /imaging dish) were co-cultured for 12h on glass-inserted imaging collagen-coated dishes. Differential interference contrast (DIC) and green channel images were acquired on a 512x512 pixel field of view, with 1.31 μ m/pixel conversion. Recordings were

performed in 30s time intervals using an inverted microscope (with 20x, 0.5 NA, objective) and a Q-imaging Fast Camera (Roper scientific), and processed by NIS Elements AR software v3.1 (Nikon). ImageJ software was used to evaluate cell trajectories and velocities. Cells were defined as stationary if they moved less than 10 $\mu\text{m}/10\text{min}$, motile if they moved more than 10 $\mu\text{m}/10\text{min}$ (Cordiglieri 2014; Kawakami 2005).

TGF β ELISA

Supernatants of DCs cultures treated for 4-8h with heat-inactivated probiotics, of DC/Teff co-cultures plated for 12h, as previously described, and of mesLN cell suspensions obtained from 2-wk treated animals with probiotics or vehicle and ex vivo cultured for 24h, were tested for TGF β protein levels by ready-to-use sandwich ELISA (eBioscience), following manufacturer's instructions.

Statistical analysis

All values were expressed as means \pm SEM. Statistical analysis was performed according to the nature of data. Normally distributed data were analyzed using one- or two-way ANOVA followed by appropriate post-hoc comparisons. $p < 0.05$ was considered statistically significant. GraphPad Prism v5.0 (GraphPad Prism, CA) was used for data elaboration and statistical analysis.

Study approval

The present studies in animals and humans were reviewed and approved by the Italian Ministry of Health (Institutional experimental code: IMP-04-11 and IMP-03-11) and were performed in respect to the Italian Principle of Laboratory Animal Care (DDL 116/92), in accordance to European Communities Council Directive 86/609/EEC and 2010/63/UE.

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Figure legends

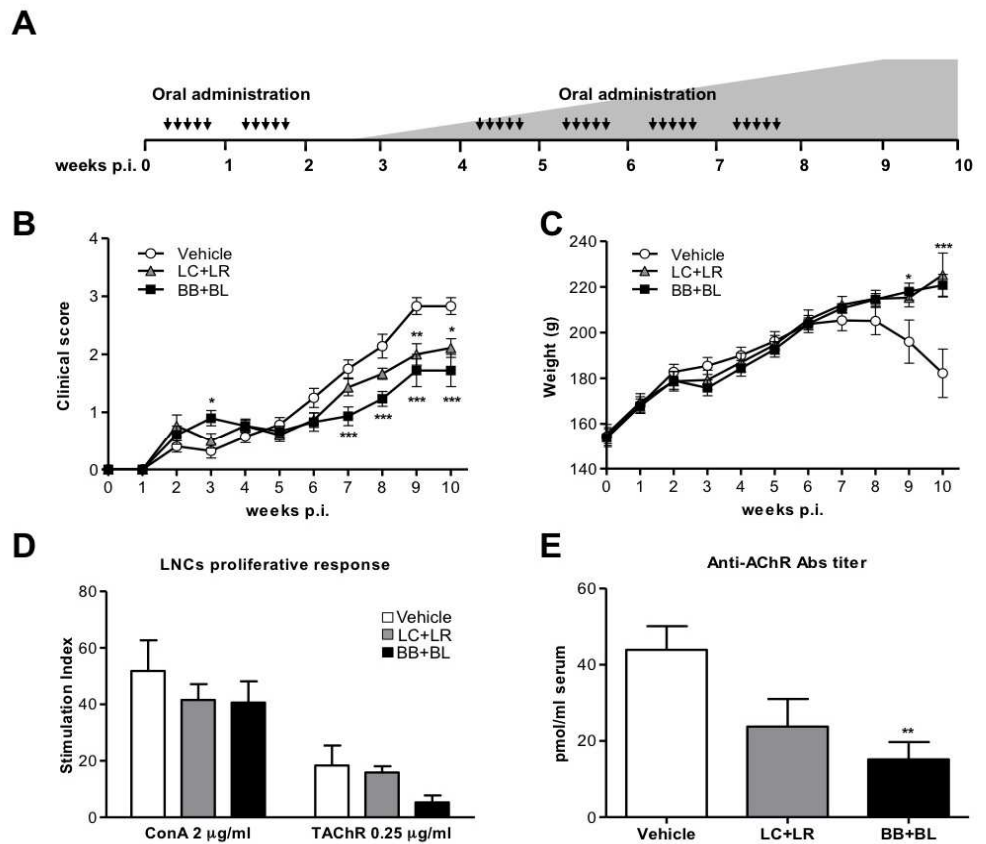


Figure 1. Combinations of newly identified probiotic strains lead to EAMG amelioration.

A) Experimental plan and treatment schedule: 2-wk preventive and 4-wk therapeutic oral administration (2.5×10^9 CFU/day; 5d/wk) in TACHr/CFA immunized Lewis rats. T=0: immunization day. **B-C)** Clinical score (**B**) and body weight change (**C**) of EAMG animals treated with LC+LR (gray triangle), BB+BL (black square), or vehicle (white circle). Mean \pm SEM values of 1 representative of 2 independent experiments, n=9 rats/group. **D)** Primary immune responses in drLNs of EAMG animals treated with LC+LR, BB+BL, or vehicle, ex-vivo stimulated with ConA and TACHr. Mean \pm SEM of 2 independent experiments, n=6 rats/group. Data of cpm values expressed as stimulation index in arbitrary units, normalized to unstimulated drLN cells. **E)** Anti-AChR Ab sera titer of EAMG animals treated with LC+LR (gray bar), BB+BL (black bar), or vehicle (white bar). Mean \pm SEM of 2 independent experiments. Statistics: 2-way-ANOVA analysis plus Bonferroni post-hoc comparisons (**B-C**) and 1-way ANOVA analysis plus Dunnett post-hoc comparisons (**D-E**) of lactobacilli- and bifidobacteria- Vs vehicle-treated animals. (*) = $p < 0.05$, (**) = $p < 0.01$, (***) = $p < 0.001$.

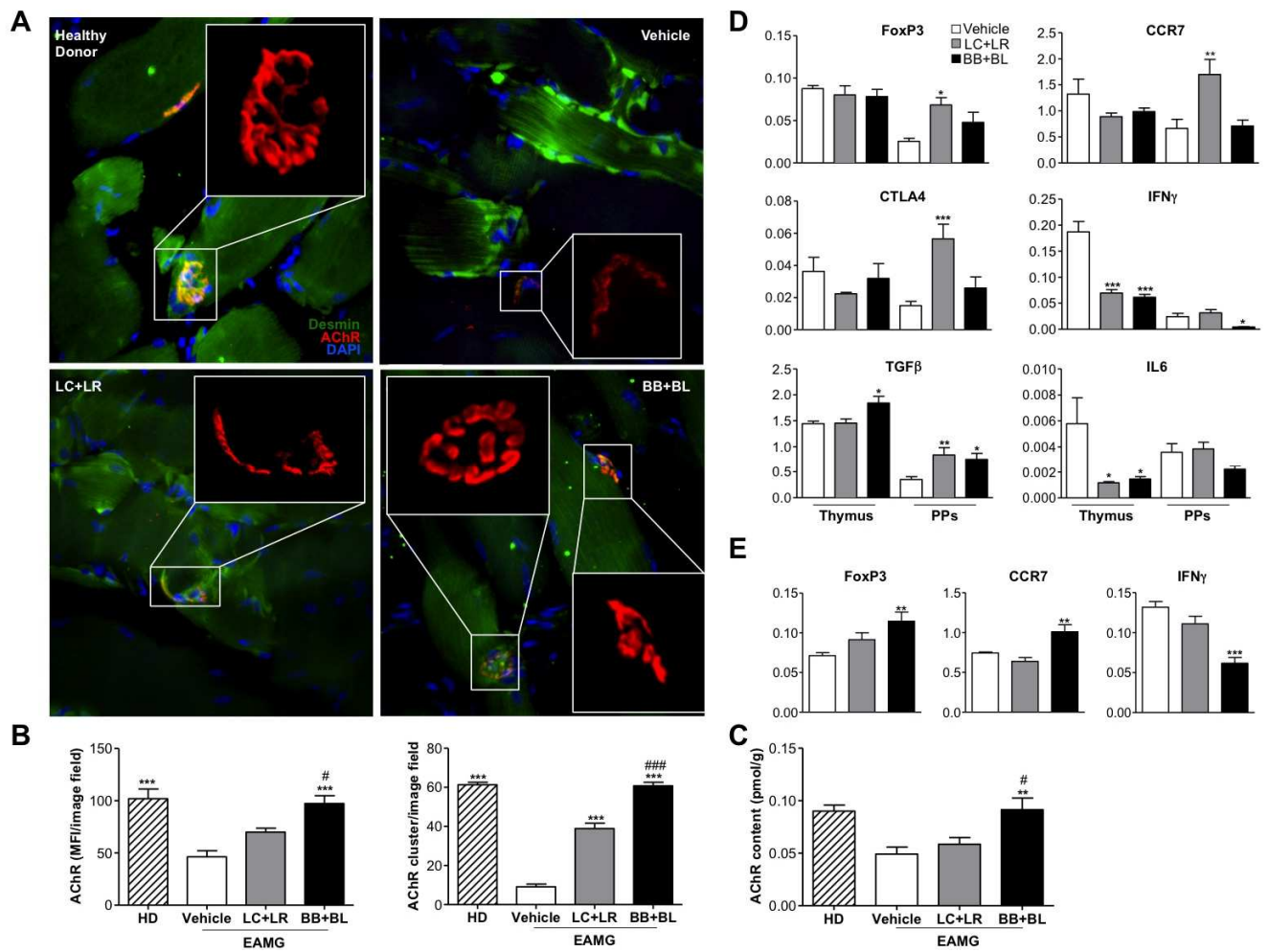


Figure 2. Bifidobacteria reduced loss in muscle AChR content and increased regulatory environment in EAMG.

A) Immunofluorescence for muscle (Desmin, green) and AChR (α BTX-Tetramethylrhodamine, red) of gastrocnemius muscle of healthy donor and EAMG rats treated with LC+LR, BB+BL or vehicle. Magnification: 50 μ m. Inserts show zoomed areas for better image of AChR clusters (magnification: 10 μ m). **B-C)** Muscle AChR quantification in healthy donor (HD; striped bars) and EAMG rats treated with LC+LR (gray bars), BB+BL (black bars) or vehicle (white bars). Mean fluorescence intensity of AChR (**B**, left graph), number of AChR clusters (**B**, right graph) in gastrocnemius muscle and total muscle AChR content (pmol/g of tissue, **C**). Mean \pm SEM of n=2 rats/group, at least 10 sections/animal analyzed for **B** and n=18 rats/group for **C**. **D-E)** qPCR analysis of thymus (**D**, left part of each graph), PPs (**D**, right part of each graph) and spleen (**E**) of

EAMG rats treated with LC+LR (gray bars), BB+BL (black bars) or vehicle (white bars). Mean \pm SEM of 2 independent experiments, n=6 rats/group. mRNA values normalized to β Actin housekeeping gene and expressed as $2^{-\Delta\text{ct}} \times 100$. Statistics: 1-way-ANOVA plus Bonferroni (B-C) or Dunnet (D-E) post-hoc comparisons. (*) = $p < 0.05$; (**) = $p < 0.01$; (***) = $p < 0.001$ Vs vehicle, (#) = $p < 0.05$, (###) = $p < 0.001$, (BB+BL) Vs (LC+LR)

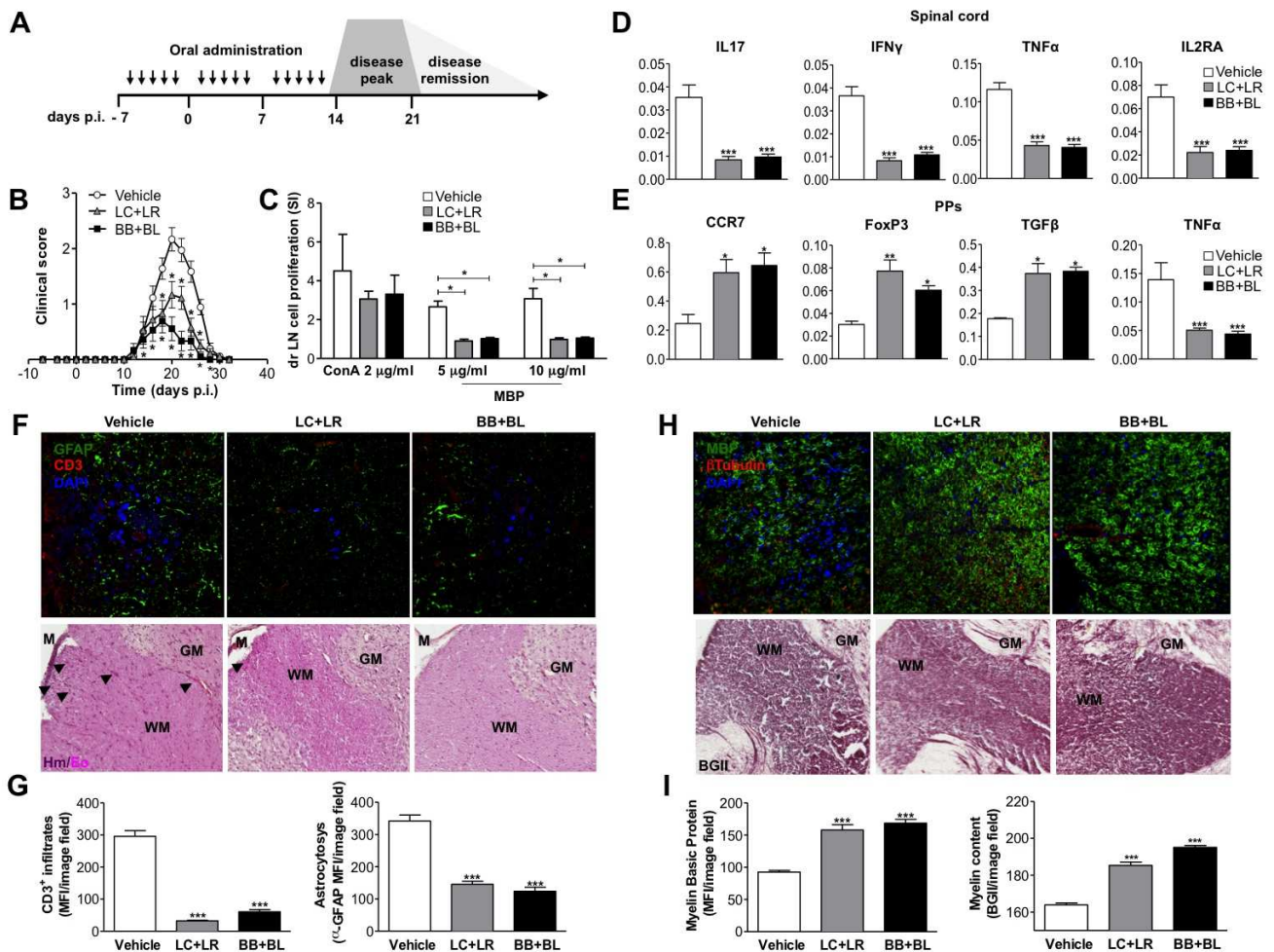


Figure 3. Combinations of newly identified probiotic strains lead to EAE amelioration.

A) Experimental plan and treatment schedule: 1-wk prophylactic and 2-wk preventive oral administration (2.5×10^9 CFU/day; 5d/wk) in MBP/CFA immunized rats. T=0: immunization day. **B)** Clinical score of EAE animals treated with LC+LR (gray triangle), BB+BL (black square), or vehicle (white circle). Representative of 3 independent experiments, n=9 rats/group. **C)** Primary responses in drLNs of EAE animals treated with LC+LR, BB+BL, or vehicle ex vivo stimulated.

Mean \pm SEM of 2 independent experiments, n=6 rats/group. **D-E** qPCR analysis of lumbar spinal cord (**D**) and PPs (**E**) of EAE rats treated as specified. Mean \pm SEM of 2 independent experiments, n=6 rats/group. mRNA values normalized to β Actin and expressed as $2^{-\Delta ct} \times 100$. **F-I** Stainings and quantifications of infiltrating Tcells (CD3, red; **F**, upper row; **G**, left), reactive astrocytes (GFAP, green; **F**, upper row; **G**, right), Hematoxyllin/Eosin stainings (**F**, lower row), oligodendrocytes (MBP, green; **H**, upper row; **I**, left), neurons (β Tubulin, red; **H**) and myelin (BG II; **H**, lower row; **I**, right) of thoracic spinal cord of EAE rats treated with LC+LR (middle images; gray bars), BB+BL (right images; black bars) or vehicle (left images; white bars). Magnification: 50 μ m. WM: white matter; GM: gray matter; M: meninges. Black arrow-heads point to immune cell infiltrates. Representative images (**F**, **H**) of 4 rats/group, 6 sections analyzed/rat (**G**, **I**). DAPI: nuclei counterstaining. MFI: mean fluorescence intensity. Statistics: 2-way-ANOVA plus Bonferroni (**B-C**) or 1-way-ANOVA plus Dunnet comparisons (**D-E**; **G**, **I**). (*) = $p < 0.05$, (**) = $p < 0.01$; (***) = $p < 0.001$ Vs vehicle-treated animals.

panels, 10 μm for middle panels. Arrow-heads point to GFP⁺ MBP-specific Tcells,. Hm/Eo: Hematoxyllin/Eosin staining. Representative images of 5 rats/group, 6 sections analyzed/rat.

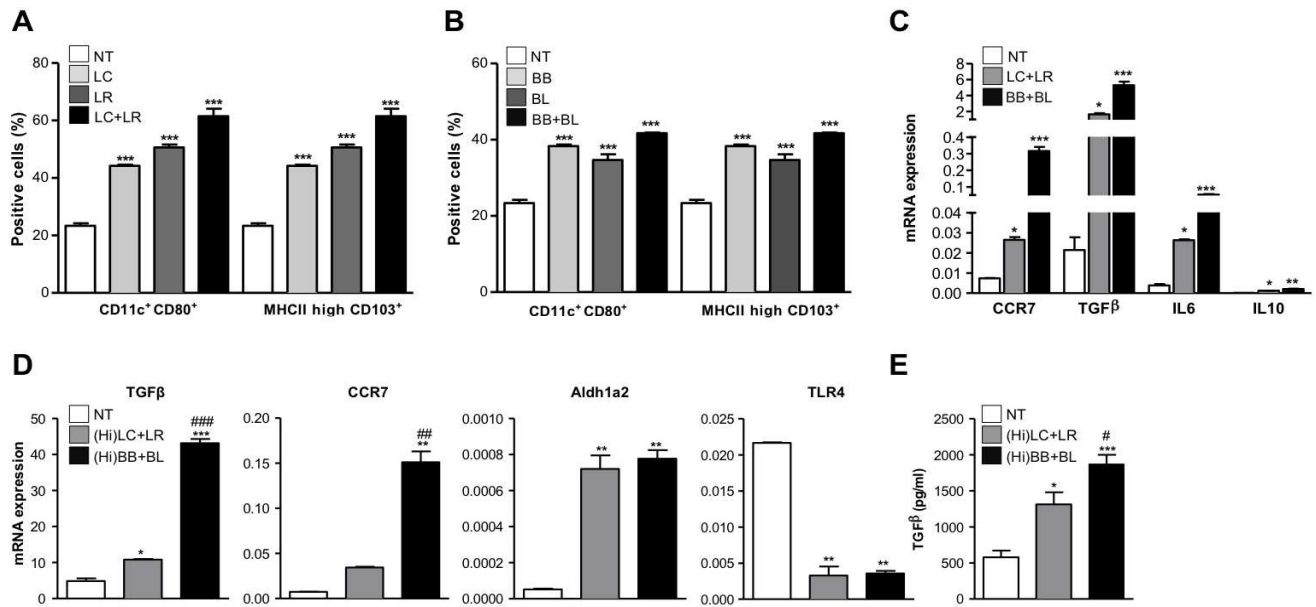


Figure 5. Both viable and heat-inactivated probiotic strains show high immune-modulatory in vitro properties on DCs

A-B) Surface marker cytofluorimetry of rat bone marrow derived DCs incubated with LC (light gray), LR (dark gray), and combination of both strains (LC+LR; black) (**A**), or with BB (light gray), BL (dark gray) and combination of both strains (BB+BL; black) (**B**), compared to untreated DCs (NT; white). Mean \pm SEM of 2 independent experiments, n=6 rats/group. **C**) qPCR analysis of rat bone marrow derived DCs incubated with LC+LR (gray) and BB+BL (black), compared to untreated DCs (NT; white). Mean \pm SEM of 2 independent experiments, n=6 rats/group. mRNA values normalized to β Actin and expressed as $2^{-\Delta\text{ct}}$ x100. **D**) qPCR analysis of immune-modulating (TGF β , CCR7, Aldh1a2) and activatory (TLR4) gene expression in DCs following 4h incubation with combinations of heat-inactivated lactobacilli (LC+LR; gray) or bifidobacteria (BB+BL; black), compared to control vehicle-treated DCs (NT; white). Mean \pm SEM of 2 independent experiments, n=6 rats/group. mRNA values normalized to β Actin and expressed as $2^{-\Delta\text{ct}}$ x100. **E**) TGF β release in supernatants of DCs following 4h incubation with combinations of

heat-inactivated LC+LR (gray) or BB+BL (black), compared to NT DCs (white). Statistics: 1-way-ANOVA plus Dunnet (A-B) or Bonferroni (C-D) post-hoc comparisons. (*) = $p < 0.05$, (**) = $p < 0.01$, (***) = $p < 0.001$ Vs NT; (#) = $p < 0.05$, (##) = $p < 0.01$, (###) = $p < 0.001$ (BB+BL) Vs (LC+LR).

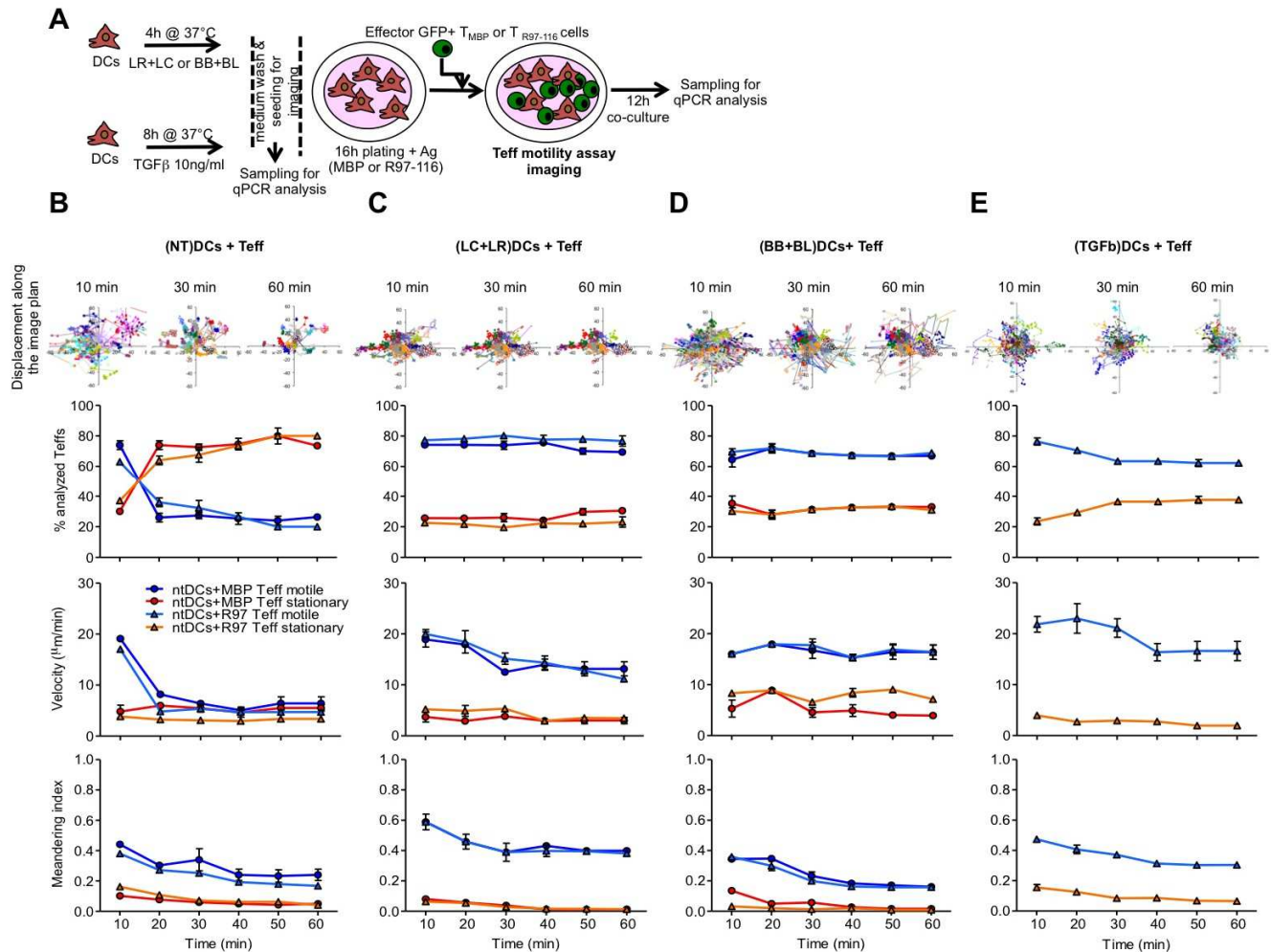


Figure 6. Inhibition of Teff activation induced by probiotic-modulated DCs.

A) Experimental scheme: DCs incubated for 4h with combinations of heat-inactivated probiotics (LC+LR; BB+BL) or for 8h with TGFβ (10ng/ml), then seeded on imaging dishes in presence of MBP or R97-116, prior to co-culture with GFP⁺ MBP-specific or R97-116 specific Teff for motility assay (1h movie) followed by 12h culture and qPCR analysis. **B-E)** Motility phenotype analysis of GFP⁺ Teff in presence of DCs vehicle-treated (B) or previously incubated with combinations of heat-inactivated lactobacilli (C) or bifidobacteria (D), or in presence of TGFβ-treated DCs (E).

Upper row: displacement of Teff along the image plan at time points 10, 30 and 60 min; second row: percentage of motile (blue for T_{MBP} eff; cyan for $T_{R97-116}$ eff) Vs stationary (red for T_{MBP} eff; orange for $T_{R97-116}$ eff) Teff over the time; third row: mean velocity ($\mu\text{m}/\text{min}$) of analyzed motile and stationary cells over the time; lower row: meandering index of motile and stationary cells. Motile cells: moving more than $10 \mu\text{m}$ in 10 min; stationary cells: defined as moving less than $10 \mu\text{m}$ in 10 min. Values shown in each graph are mean quantifications \pm SEM of triplicate time-lapse videos for each condition with T_{MBP} and triplicate videos with $T_{R97-116}$ with at least 225 T_{MBP} and 225 $T_{R97-116}$ effector cells analyzed for each time stack (time-lapse: 30 seconds; 121 time points/video). For TGF β -treated DC condition, only R97-116 specific Teff were employed (triplicate videos with 225 Teff analyzed). No differences were observed between T_{MBP} and $T_{R97-116}$ in the motility behavior.

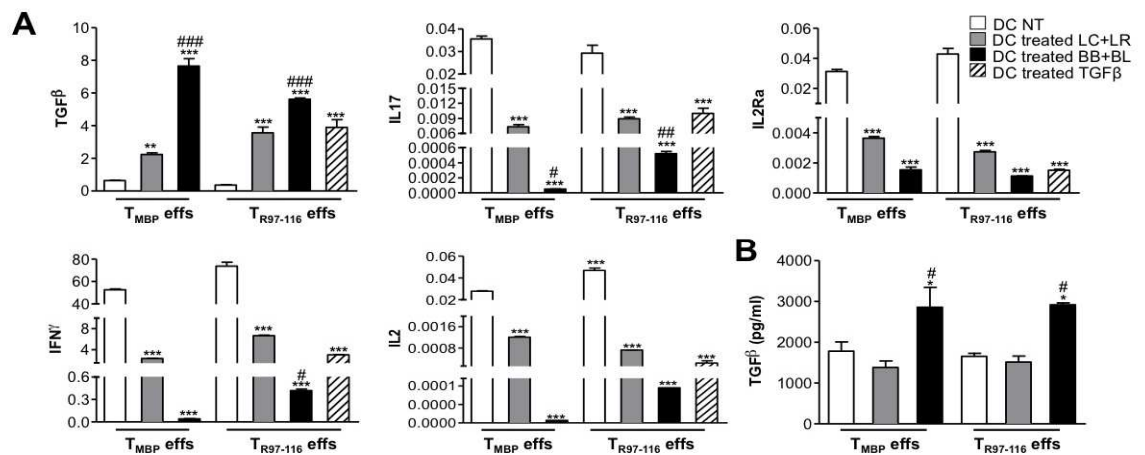
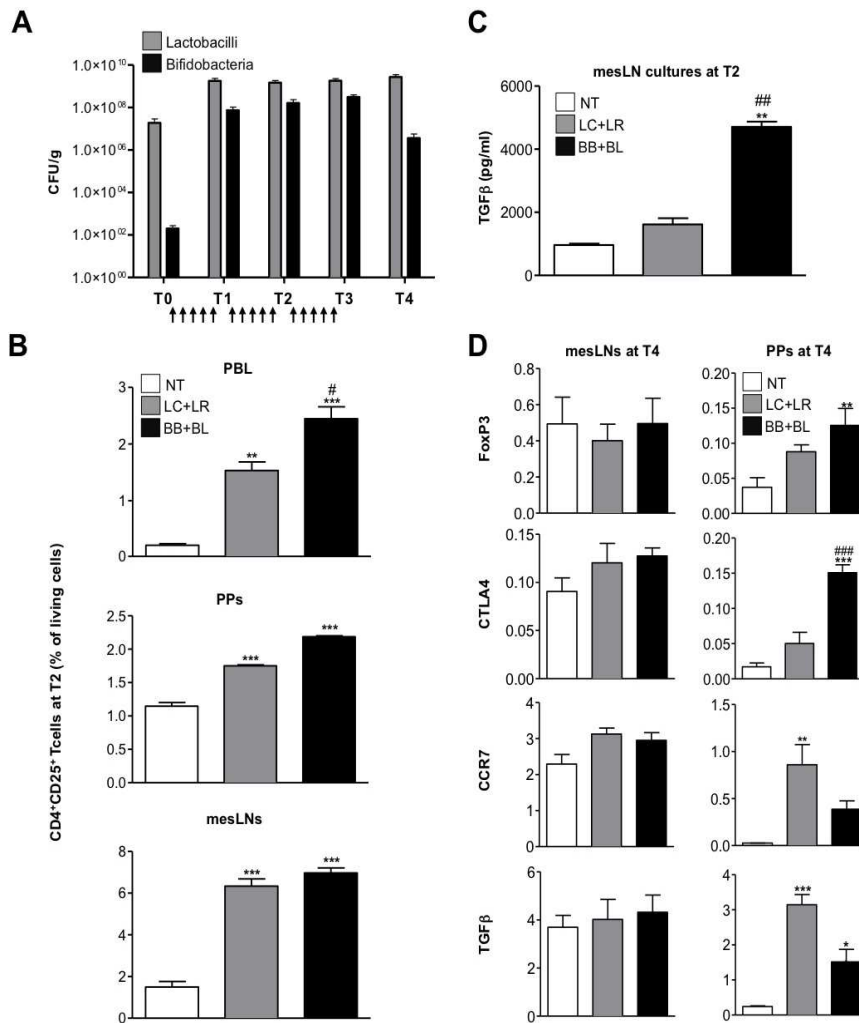


Figure 7. Inhibition of Th1/Th17 inflammatory milieu by probiotic-modulated TGF β -inducing DCs

A) Cytokine qPCR analysis after 12h co-culture of GFP⁺ antigen-specific Teff in presence of antigen-pulsed DCs previously incubated with heat-inactivated probiotic combinations (LC+LR, gray; BB+BL, black), TGF β (striped) or vehicle (NT, white), as represented in Figure 6. Mean \pm SEM of 2 independent experiments, (n=3/group with T_{MBP} ; n=3/group with $T_{R97-116}$). Data show mRNA values normalized to β Actin and expressed as $2^{-\Delta ct} \times 100$. Statistics: 1-way-ANOVA plus

Dunnet post-hoc comparisons: **B)** TGF β release in supernatants of 12h co-culture of GFP⁺ antigen-specific Teff in presence of antigen-pulsed DCs previously incubated with heat-inactivated probiotic combinations (LC+LR, gray; BB+BL, black), or vehicle (NT, white) Statistics: 1-way-ANOVA plus Dunnet (**A**) or Bonferroni (**B**) post-hoc comparison. (*) = p<0.05, (**) = p<0.01, (***) = p<0.001 Vs NT; (#) = p<0.05; (##) = p<0.01; (###) = p<0.001 (BB+BL) Vs (LC+LR).

Supplementary material

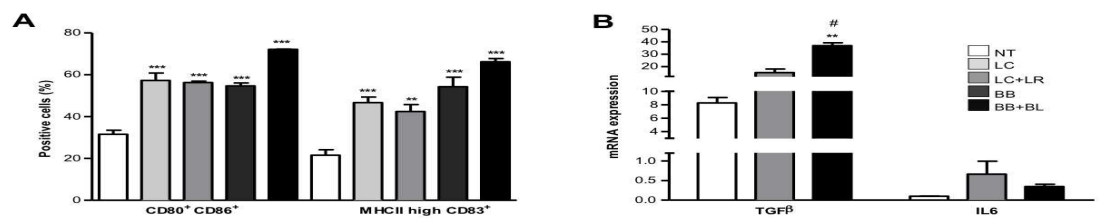


Supplementary Figure 1. Probiotic immune-modulatory in vitro properties and in vivo gut colonization

A) Amount of total lactobacilli and bifidobacteria in fecal samples, as analyzed via decimal counts of viable bacteria in selective culturing media over time of administration and wash out (T=4; gray: LC+LR; black: BB+BL). Representative data of 2 independent experiment, n=3/group

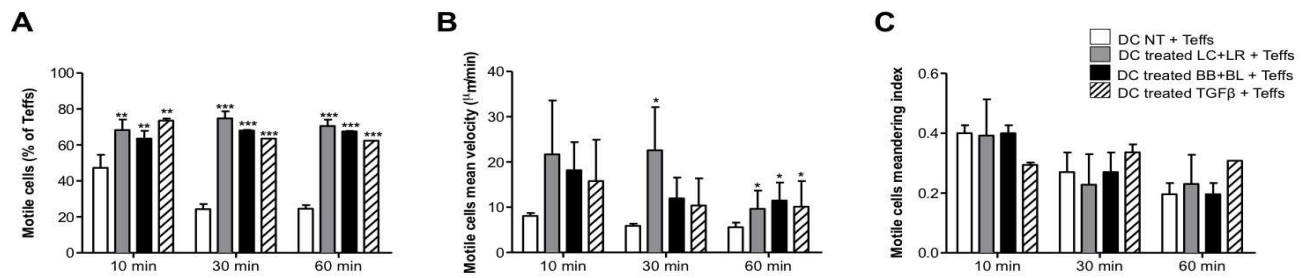
B) Percentages of CD4⁺CD25⁺ Treg at T2 in PBL, PPs and mesLNs of animals treated with LC+LR

(gray), BB+BL (black) or vehicle (white). **C**) TGF β protein levels in the supernatants of 24h cultured mesLN cells from animals following 2-wk treatment with LC+LR (gray), BB+BL (black) or vehicle (white). **D**) qPCR analysis of locally-altered immune-modulating genes in mesLNs (left panel) and PPs (right panel) following probiotic treatment at T=4 (gray: LC+LR; black: BB+BL; white: vehicle). Mean \pm SEM of 2 independent experiments, n=6 rats/group. Data show mRNA values normalized to β Actin housekeeping gene and expressed as $2^{-\Delta ct} \times 100$. Statistics: 1-way-ANOVA plus Dunnett (**B, C, D**) post-hoc comparisons: (***) = $p < 0.001$, (**) = $p < 0.01$ Vs vehicle; (#) = $p < 0.05$, (##) = $p < 0.01$, (###) = $p < 0.001$ (BB+BL) Vs (LC+LR).



Supplementary Figure 2. Newly identified probiotic strains induce immune-regulatory phenotype on human DCs

A-B) Human monocyte-derived DCs incubated with LC (light gray) and BB (dark gray), or with combinations of LC+LR (steel gray) and BB+BL (black) compared to untreated DCs (NT; white). Surface marker cytofluorimetry (**A**) and qPCR analysis (**B**). Mean \pm SEM of 2 independent experiments, n=6/group. Data show mRNA values normalized to GAPDH housekeeping gene and expressed as $2^{-\Delta ct} \times 100$. Statistics: 1-way-ANOVA plus Bonferroni post-hoc comparisons: (***) = $p < 0.001$; (**) = $p < 0.01$ Vs NT; (#) = $p < 0.05$ (BB+BL) Vs (LC+LR).



Supplementary Figure 3. Inhibition of Teff by immune-modulated DCs.

A-C) Statistical analysis and comparisons among co-cultures of Teff (both MBP- and R97-116 specific) in presence of DCs treated with combinations of LC+LR (gray), BB+BL (black), TGFβ (striped) or untreated DCs (NT, white), as shown in Figure 6. Graphs show percentages (**A**), mean velocity ($\mu\text{m}/\text{min}$, **B**) and meandering index (**C**) of motile Teff at time point 10, 30 and 60 min of imaging analysis. Values shown in each graph are mean quantifications \pm SEM of six time-lapse videos for each condition with at least 450 Teff analyzed for each time stack (time-lapse: 30 seconds; 121 time points/video). For TGFβ-treated DC condition, only $T_{R97-116}$ eff was employed (triplicate videos with 225 Teff analyzed). Statistics: 2-way-ANOVA plus Bonferroni post-hoc comparisons: (*) = $p < 0.05$; (**) = $p < 0.01$; (***) = $p < 0.001$ Vs co-culture of Teff with (NT)DCs.

Supplementary Video 1.

Rat T_{MBP} effs form stable contacts with MBP-presenting DCs and get activated. Time-lapse video-microscopy of rat $GFP^{+/-} T_{R97-116}$ eff (green) in co-culture with rat R97-116-loaded DCs and 10 $\mu\text{g/ml}$ R97-116 Ag. Overlaid images of green fluorescence and differential interference contrast (DIC) channels. Observation time: 60 min. 30-sec intervals. Stack reproduction rate: 5 frames/sec. Shown is a cropped detail of one representative movie from 4 independent recordings of co-culture condition. Magnification: 10 μm .

Supplementary Video 2.

Pre-incubation with combination of heat-inactivated LC+LR induces immune-modulatory phenotype on DCs leading to dampening of Teff/DC R97-116-specific stable contacts. Time-lapse video-microscopy of rat $GFP^{+/-} T_{R97-116}$ eff (green) in co-culture with rat R97-116-loaded DCs and 10 $\mu\text{g/ml}$ R97-116 Ag. Overlaid images of green fluorescence and DIC channels. Observation time: 60 min. 30-sec intervals. Stack reproduction rate: 5 frames/sec. Shown is a cropped detail of one representative movie from 4 independent recordings of co-culture condition. Magnification: 10 μm .

Supplementary Video 3.

Pre-incubation with combination of heat-inactivated BB+BL induces immune-modulatory phenotype on DCs inhibiting Teff/DC R97-116-specific stable contacts and Teff activation. Time-lapse video-microscopy of rat $GFP^{+/-} T_{R97-116}$ eff (green) in co-culture with rat R97-116-loaded DCs and 10 $\mu\text{g/ml}$ R97-116 Ag. Overlaid images of green fluorescence and DIC channels. Observation time: 60 min. 30-sec intervals. Stack reproduction rate: 5 frames/sec. Shown is a cropped detail of one representative movie from 4 independent recordings of co-culture condition. Magnification: 10 μm .

Supplementary Video 4.

Pre-incubation with $TGF\beta$ induces immune-modulatory phenotype on DCs inhibiting Teff/DC contacts and Teff activation. Time-lapse video-microscopy of rat $GFP^{+/-} T_{R97-116}$ eff (green) in co-culture with R97-116-loaded DCs and 10 $\mu\text{g/ml}$ R97-116 Ag. Overlaid images of green fluorescence and DIC channels. Observation time: 60 min. 30-sec intervals. Stack reproduction

rate: 5 frames/sec. Shown is a cropped detail of one representative movie from 4 independent recordings of co-culture condition. Magnification: 10 μm .

***Chapter 6 - New probiotic candidates VS
biogenic amines: in-vitro screening criteria***

New probiotic candidates VS biogenic amines: in-vitro screening criteria

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Introduction

Biogenic amines (BA), mainly tyramine, histamine, putrescine, cadaverine, are low molecular weight organic bases formed by the quick broken down of food proteins in the body by enzymes, such as MAO (monoamine oxidase-A), which render them harmless. Toxic effects of BA on human body can develop through both increased availability, due to high amounts of ingestion by foods, and/or impaired BA degradation, due to increased sensitivity of individuals. A pathological deficiency of amine oxidase activities as well as the excessive consumption of ethanol and some drugs (Zimatkin and Anichtchik, 1999; Horton *et al.*, 2005) may lead to high levels of BA in the organism (Kuefner *et al.*, 2004). Several toxicological problems related to high levels of BA were reported (Marinè-Font *et al.*, 1995) such as effects derived from their vasoactive properties of histamine and tyramine, as well as palpitations, headaches, vomiting and hypertension (Silla Santos, 1996; Lonvaud-Funel, 2001).

Apart from the reduction of BA accumulation in foods following the choice of the right starter culture, envisaged by EFSA in 2011, the control of BA in human body could be realized mainly through a reduced exogenous ingestion of BA-rich fermented foods. Biogenic amines in effect occur in different kinds of food, such as cheese, wine, beer, fishery products and other fermented foods (Halász *et al.*, 1994). The highest mean values for the sum of biogenic amines were retrieved in 'fish sauce' (582 - 588 mg/kg), fermented vegetables (375 – 390 mg/kg), cheese (177 – 334 mg/kg) and fermented sausages (281 – 283 mg/kg) (EFSA opinion, 2011). The active growth of several bacteria and proteolysis during sausage fermentation, makes this environment particularly favourable to biogenic amine formation (Bover-Cid *et al.*, 1999). Moreover, the catabolism of amino acids carried by LAB and the resulting high concentration of BA can affect the quality of fermented foods and their organoleptic properties (Verges *et al.*, 1999; Palacios *et al.*, 2005).

In fermented foods, BA are mainly generated by decarboxylation of the corresponding amino acids through specific enzymes of Lactic Acid Bacteria (LAB) and, for this reason, testing amino acid decarboxylation activities of LAB, before using them as starter cultures for fermentation, is of great relevance.

LAB are usually able to produce histamine via histidine decarboxylase (Lonvaud-Funel and Joyeux, 1994), tyramine via tyrosine decarboxylase (Lucas *et al.*, 2003) and putrescine via ornithine decarboxylase (Marcobal *et al.*, 2004).

BA are also endogenously produced in the human gut by the microbiota that degrade proteins and amino acids abundantly available in this natural environment (de Palencia *et al.*, 2011). The actual relevance for public health of BAs directly produced in the intestine is presently unclear and not proven (EFSA opinion, 2011) despite a current strong debate about the impact of high-protein dietetic regimens on human health and the potential deleterious effects of this diet on gut health, with special reference to red and processed meat, and increased risk to develop cancer (IARC Monographs, vol. 114, October 2015).

The production of BA has been associated with some groups of microorganisms. For instance, putrescine and cadaverine production is frequently found in enterobacteria, a bacterial group for which presence and distribution of amino acid decarboxylase activity has been largely studied. Very few information regarding the production of BA are available for LAB. Some species of lactobacilli, mainly *Lactobacillus buchneri* and *L. curvatus*, have been recognized to produce histamine and tyramine. No data are currently available about bifidobacteria. Within microbial groups, the capacity to produce BA has been recognised, however, as a strain-specific feature (Coton and Coton, 2009; Lucas *et al.*, 2005; Marcobal *et al.*, 2006).

Different screening methods are available to detect amino acid decarboxylase activity in bacteria. Qualitative methods usually involve the use of a precursor amino acid and a medium containing a pH indicator (Moeller 1954; Choudhury *et al.*, 1990), its color change indicates the production of a more alkaline BA by the cultured bacteria from the precursor amino acids. The limit of this method is the possibility to create false positive results, due to the formation of alkaline compounds other than BA and false negatives results due to the fermentation activity of many bacteria that leads to the formation of acids compounds. Therefore other methods were developed to detect bacteria able to generate BA in the environment. In this context PCR has become an important method for the rapid and specific detection of the target genes. Marcobal *et al.* (2005) described a multiplex PCR based on primers targeting genes encoding amino acid decarboxylase of LAB as potential BA producers.

Despite the previous observations, recent reports considered the role of LAB as BA degraders. In fact, LAB isolated from fermented foods have been proven to degrade BA through the production of amine oxidase enzymes. Garcia-Ruiz *et al.* (2011) tested 85 LAB strains isolated from wine. The greatest BA-degrading ability was exhibited by 9 strains belonging to the *Lactobacillus* and *Pediococcus* genera, and most of them were able to simultaneously degrade at least two of the three studied BA. Capozzi *et al.* (2012) postulated the use of two strains of *L.*

plantarum for the degradation of BA in wine based on their ability to catabolize tyramine and putrescine leading to more than 30% breakdown in culture media. In 2001, Fadda *et al.* isolated from meat some strains of *L. casei* and *L. plantarum* which have been proved to be able to degrade tyramine.

The control of BA-producers by the antagonistic effect of BA-degraders in natural environments as well in the human body could be of great interest in order to control harmful bacterial population in the gut and to limit the overproduction of BA due to the increased ingestion of proteins of animal origin. This could open the way to the use of probiotic-based supplements with improved activity in BA degradation.

During the study presented in the manuscript, a pool of newly isolated lactobacilli and bifidobacteria, previously characterized for their in-vitro probiotic properties, were screened taking into consideration the dualism between production and degradation that lactobacilli encounter with respect to BA metabolism. Firstly they were investigated for the undesired feature of the ability to synthesise BA (Capozzi *et al.*, 2012; Landete *et al.*, 2007) and then for the valuable ability of BA degradation through the activity of specific enzymes.

Materials and methods

Strains and culture conditions

Strains of lactobacilli and bifidobacteria isolated from fecal samples or vaginal swabs were grown at 37°C in MRS broth (BD Difco, USA) supplemented, in the case of bifidobacteria, with L-cysteine hydrochloride (Sigma-Aldrich, Germany) at a concentration of 0,5 g/l. Cultures were incubated for 24-48 hours in anaerobic conditions. Strains were cultured on plates of agar MRS and TOS mupirocin 100 µg/ml media for lactobacilli and bifidobacteria, respectively. Plates were incubated at 37°C for 48-72 hours in anaerobic conditions. The enzymatic activity of the enzymes involved in BA degradation was detected by growing the strains on culturing media supplemented with 100 µg/ml of each BA such as histamine, tyramine, etc. Cultures, incubated for 16 hours at 37°C in anaerobic conditions, were then centrifuged and submitted to the preparation described below. Table 1 lists the screened strains.

DNA extraction and PCR primers and procedure

For use in Polymerase Chain Reaction (PCR) DNA from bacteria was extracted using Whatman™ FTA™ CloneSaver™ technology (GE Healthcare Life Sciences, UK).

In order to identify strains able to produce one or more biogenic amines, PCR primers targeting amino acid decarboxylase genes were used as described by Coton *et al.* (2010). Primer sequences and their targets are listed in Table 2.

L. reuteri DSM 20016^T was included as positive control for histamine production. PCR was conducted in a final volume of 50 µl. Amplification reaction mixture contained 50 ng of bacterial DNA, one of the available pairs of primers depending on the gene to be amplified (0,12 µM of HDC3/HDC4 for histidine decarboxylase, 0,2 µM of TD2/TD5 for tyrosine decarboxylase, 0,8 µM of ODC1/ODC2 for ornithine decarboxylase or 0,8 µM of AgD1/AgD2 for agmatine deaminase) and MegaMix (Microzone, UK).

The reactions were performed in an automated SimpliAmp thermal cycler (Life Technologies, USA) using the following cycling parameters: 95°C for 5 minutes followed by 35 cycles of 95°C 1min, 52°C 1 min, 72°C 1min 30 s with a final extension step of 5 min at 72°C (Coton *et al.*, 2010). Amplified products were resolved on a 0,8% (wt/vol) agarose gel (Segetic, Germany) and stained with ethidium bromide. The amplicon expected for the reference strain *L. reuteri* DSM 20016^T was about 400 bp fragment of *hdc* gene.

Protein extraction from probiotic cells

The ability to degrade BA by oxidation of the same was tested according to the method of Callejòn *et al.*, 2014 with some modifications. Strains were grown overnight in 50 ml of medium supplemented with BA as described in *Strains and culture conditions*. The cultures grown were centrifuged to recover the bacterial pellet and washed twice with 25 ml of phosphate buffer 50 mM pH 7.4. The pellet was resuspended in 0.5 ml of the same buffer that was previously added with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Germany) and transferred into eppendorf tubes of 1.5 ml containing glass beads with a diameter of 100 µm. Cells were disrupted in FastPrep-24 (MP, USA) by 4 cycles of 45 seconds each, alternated to incubation in ice for 5-10 minutes between one cycle and the other. Cell extracts were centrifuged at 13000 rpm for 15 min to precipitate the pellet and the supernatant was recovered and kept at -20°C until use.

Quantification of the extracted proteins

Extracted proteins were quantified using Pierce BCA Protein Assay Kit (Thermo SCIENTIFIC, USA) following the instruction of the supplier. A set of serial dilutions of BSA (internal control) and the BCA Working Reagent were prepared and use to design a standard line. Test samples incubated at 37°C for 30 minutes were submitted to spectrophotometric lecture at O.D. 562 nm in order to measure the absorbance. A standard curve was designed by plotting O.D. measurement for each BSA standard vs its known concentration in µg/ml. Final protein concentration of the tested samples was indirectly calculated based on their O.D. value by using the standard curve as reference.

Enzymatic assay for diamine oxidase

20 µl of each protein extract was mixed with 10 µl of loading buffer (10% glycerol, 50 mM Tris-HCl pH 6,8, bromophenol blue 0,02%). 20 µl of this mix was loaded on a stacking 4% polyacrylamide gel and separated in a 8% polyacrylamide gel using an electrophoretic buffer consisting of Tris-glycine (25 mM Tris base, 192 mM glycine), as proposed by Callejòn *et al.* (2014). The purified enzyme Laccase from *Trametes Versicolor* (Sigma-Aldrich, Germany) was used as positive control in the gel. At the end of the electrophoretic run the gel was placed in sodium phosphate buffer (50mM, pH 7,4) containing 1 mM of each biogenic amine, for 15 min. The solution was then removed and replaced by a new solution consisting of the same buffer containing horseradish peroxidase (1000 U/l) and diaminobenzidine 0,25 mM (DAB). The ability to degrade BA was revealed by the appearance of a brown color, due to the formation of a precipitate as a result of DAB oxidation, on the reference band after 1-2 hours of staining.

Results

BA synthesis by novel probiotic lactobacilli

The PCR approach proposed by Coton *et al.* (2010) was applied to the detection of the genes coding for amine decarboxylase like tyrosine decarboxylase (*tyrdc*) for tyramine, ornithine decarboxylase or agmatine deaminase (*odc* and *agdi*) for putrescine and histidine decarboxylase (*hdc*) for histamine. Almost all the isolated strains were negative for the detection of the 4 genes. Only *L. vaginalis* CBA-L88 showed two amplicons of about 400 bp and

600 bp, indicating that this strain putatively possess the genes for the production of histamine *via* histidine decarboxylase (*hdc+*) and putrescine *via* agmatine deaminase (*agdi+*).

Figures 1 and 2 present the images of the electrophoretic gels carrying the amplicons obtained for the control and the tested strains. This strain was therefore excluded from the subsequent screening phase since it appears not suitable for safety reasons to be used as probiotic in viable form. Sanders et al. (2010) observed that the addition of probiotic organisms to foods should take biogenic amine producing capacity into account if opportunity for production and substrate availability are conducive to such activity.

BA degradation by novel probiotic lactobacilli

Proteins extracted from the viable cells of the tested strains were quantified and normalised to a final concentration of about 10 mg/ml. These extracts were used in diaminobenzidine (DAB) assay in the presence of a mixture of histamine, tyramine and putrescine. Strains positive to the detection of the amino oxidase, as well as the control enzyme from *Trametes versicolor*, showed a brown precipitate indicating the link of the chromogen to the substrate. Results shown in Figure 3 indicate that one strain *L. rhamnosus* CBA-L140 possess the enzyme for the degradation of BA while for all the other tested strains showed negative reactions. Another *L. rhamnosus* strain, CBA-L140, showed a positive reaction but different in size if compared to positive control since it putatively carries a different enzyme responsible for the degradation of BA.

Conclusions

The production of BA represents an issue of great interest due to their detrimental impact on human health, proportional to their gut concentration. The problem of the accumulation of BA mainly concerns fermented products and food with high protein content because different gut bacteria are able to synthesize BA via decarboxylation of the amino acids. Moreover the lack of information about the ability of the strains to synthesize BA leads to neglect this issue and to the use of BA-forming strains as starter in food fermentation. The investigation of the ability of bacterial strains to synthesize BA leads to increased knowledge of the same and so to a more conscious use in food preparations. It is necessary to confirm the inability of the strain to synthesize BA before considering it as safe and therefore suitable to be used as a starter culture as well as a probiotic for which the presence of genes coding for amine decarboxylase is

considered a safety concern. PCR method to detect genes involved in the synthesis of the BA represents a rapid and specific tool for the selection of strains with decarboxylase activity, other than the conventional approach based on metabolic tests on supernatants of bacteria cultured on BA.

On the other hand, the potential for BA breakdown should be considered an important feature among starter and probiotic strains, since some LAB show oxidase activities. This aspect should represent an additional criterion in the selection of strains for food fermentation and/or food supplements. The identification of one or more *Lactobacillus* and/or *Bifidobacterium* strain able to degrade BA in-vitro as well as its subsequent genetic and functional characterization could open the way to the use of a probiotic strain as food supplement in hypocaloric, high protein feeding regimens.

Legends

Table 1: bacterial strains used in the present work.

Table 2: primers used in the PCR targeting BA-production genes. *hdc*, histidine decarboxylase; *tyrdc*, tyrosine decarboxylase; *odc*, ornithine decarboxylase; *agdi*, agmatine deaminase.

Figure 1: PCR with primers HDC3/HDC4 to detect the *hdc* gene. A 200 bp marker (Invitrogen, USA) was included (lane 1).

Figure 2: PCR with primers AgD1/AgD2 to detect the *agdi* gene. A 200 bp marker was included (lane 1).

Figure 3: amino degradative activity showed by LAB extracts as a result of electrophoresis on polyacrylamide gel and staining with DAB.

Table 1

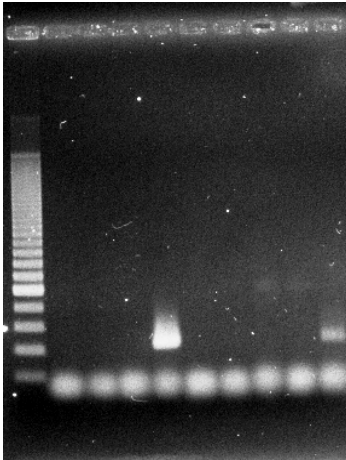
Bacterial strains screened for BA production	Bacterial strains screened for BA degradation
<i>L. rhamnosus</i> CBA-L142	<i>L. rhamnosus</i> CBA-L92
<i>L. rhamnosus</i> CBA-L140	<i>L. rhamnosus</i> CBA-L143
<i>L. rhamnosus</i> CBA-L143	<i>L. fermentum</i> CBA-L147
<i>L. vaginalis</i> CBA-L88	<i>L. rhamnosus</i> CBA-L142
<i>B. breve</i> CBA-B104	<i>L. fermentum</i> CBA-L146
<i>B. breve</i> CBA-B171	<i>L. gasseri</i> CBA-L72
<i>L. reuteri</i> commercial strain	<i>L. rhamnosus</i> CBA-L140
<i>L. reuteri</i> DSM 17938	<i>L. paracasei</i> CBA-L144
<i>L. reuteri</i> DSM 20016	<i>L. acidophilus</i> CBA-L63
	<i>L. paracasei</i> CBA-L101
	<i>L. paracasei</i> CBA-L145

Table 2

Target gene	Primer	Sequence	Product (bp)	Reference
<i>hdc</i>	HDC3	GATGGTATTGTTTCKTATGA	435	Coton <i>et al.</i> , 2010
<i>hdc</i>	HDC4	CCAAACACCAGCATCTTC	435	Coton <i>et al.</i> , 2010
<i>tyrdc</i>	TD2	ACATAGTCAACCATRTTGAA	1133	Coton <i>et al.</i> , 2010
<i>tyrdc</i>	TD5	CAAATGGAAGAAGAAGTAGG	1133	Coton <i>et al.</i> , 2010
<i>odc</i>	ODC1	NCA YAARCAACAAGYNGG	900	Coton <i>et al.</i> , 2010
<i>odc</i>	ODC2	GRTANGGNTNNGCACCTTC	900	Coton <i>et al.</i> , 2010
<i>agdi</i>	AgD1	CAYGTNGAYGGHSAAGG	600	Coton <i>et al.</i> , 2010
<i>agdi</i>	AgD2	TGTTGNGTRATRCAGTGAAT	600	Coton <i>et al.</i> , 2010

Figure 1

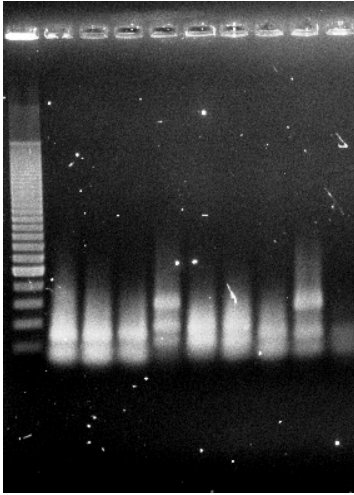
1 2 3 4 5 6 7 8 9 10



1. Molecular marker weight 200 bp (Invitrogen, USA)
2. *L. rhamnosus* CBA-L142
3. *L. rhamnosus* CBA-L140
4. *L. rhamnosus* CBA-L143
5. *L. vaginalis* CBA-L88
6. *B. breve* CBA-B104
7. *B. breve* CBA-B171
8. *L. reuteri* commercial strain
9. *L. reuteri* DSM 17938
10. *L. reuteri* DSM 20016

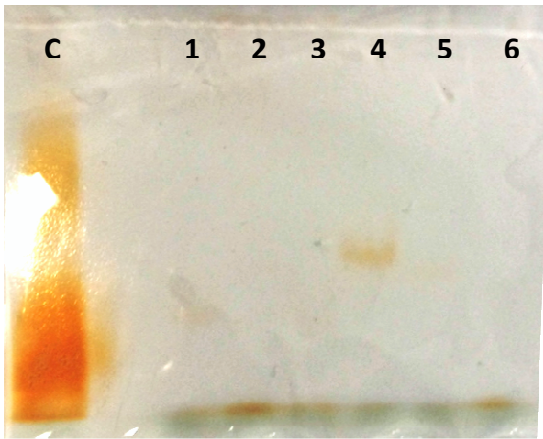
Figure 2

1 2 3 4 5 6 7 8 9 10



1. Molecular marker weight 200 bp
2. *L. rhamnosus* CBA-L142
3. *L. rhamnosus* CBA-L140
4. *L. rhamnosus* CBA-L143
5. *L. vaginalis* CBA-L88
6. *B. breve* CBA-B104
7. *B. breve* CBA-B171
8. *L. reuteri* commercial strain
9. *L. reuteri* DSM 17938
10. *L. reuteri* DSM 20016

Figure 3



- C. Positive control: Laccase from *Trametes versicolor*
- 1. *L. paracasei* CBA-L144
- 2. *L. acidophilus* CBA-L63
- 3. *L. paracasei* CBA-L145
- 4. *L. rhamnosus* CBA L-140
- 5. *L. rhamnosus* CBA L-142
- 6. *L. paracasei* CBA-L101

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Chapter 7: Summary and concluding remarks

Nowadays, the market of food supplements is particularly rich and varied. In recent years, the spread of probiotics containing *Lactobacillus* or *Bifidobacterium* mostly in association with other bacteria, is considerably increased.

The demand for health by the population has undergone a sharp increase in recent years in both quantitative and qualitative terms, consumers are becoming in fact more knowledgeable and attentive to the issues of health and prevention.

An increasing number of functional foods are enriched with probiotics and for consumers is becoming increasingly important to get the information necessary to know how to make the best choices.

For these reasons it is necessary an intensive research in order to respond to the needs of the individuals in term of health and prevention.

During my PhD thesis I was in charge for the screening of new potential probiotic strains by combining conventional approach to newly designed platforms especially targeted towards health-promoting applications. I initially focused on the so-called "conventional screening" aimed to the isolation of new bacterial strains of lactobacilli and bifidobacteria and to assess the safety of the same for use in human food (second signs of national and international regulatory bodies). This first part of the work, in addition to safety, was aimed to assess the efficacy of the bacterial strains: for use in humans is in fact important that the strains are able to survive in the acidic environment of the stomach and able to survive and multiply in the intestine even in the presence of bile salts.

Bacterial strains found to possess these features were then subjected to a more specific screening, depending on the application to which they will be destined. They will therefore be intended to a process of "selection funnel" aimed at the realization of a series of *in vitro* tests to assess their applicability as probiotics for the promotion of human health.

About that, it is important to emphasize that while some effects of probiotics, such as their ability to reduce intestinal disorders, are well documented; for other conditions are not available strong evidences that demonstrate the efficacy of the same. Moreover, the effects are "strain-specific" and must not be extended to other probiotics of the same genus or species.

I'm so focused on new areas of application, and I assessed the applicability of the selected strains in food or pharmaceutical field.

A first activity of functional characterization of new probiotic strains was carried out in collaboration with the Department of Food Science of the University of Udine and was aimed to evaluate the potential use of new strains in the food supplementation of patients that follow a high-protein diets to reduce the risk of intestinal accumulation of biogenic amines (metabolic application). This activity has allowed me to identify a strain of *Lactobacillus* able to synthesize biogenic amines and one strain with alleged amino degradative activity that must be confirmed soon. I would like in fact to continue the characterization of this best suitable candidate as BA degraders, by quantifying BA in culture medium incubated with and without the strain, *via* high-performance liquid chromatography (HPLC)-based methods or alternative developed methods and therefore ranking the degradative activity expressed by this selected strain.

Another screening activity is derived from the collaboration between AAT Srl and the Neurological Institute "Carlo Besta", which had as its main objective the study of the immunoregulatory effects of new strains of lactobacilli and bifidobacteria. Individual strains or combinations of probiotics were evaluated and are resulted good promising candidates for the treatment of inflammatory and autoimmune diseases. Among these were taken into account the myasthenia gravis and multiple sclerosis, diseases of great interest because of their highly debilitating impact on daily life.

The treatment of dendritic cells with different combinations of probiotics led to a strong immune modulation and oral administration showed therapeutic properties, thereby improving the clinical symptoms in both models used for the study of the two diseases.

In particular, we focused on two newly identified strains: *L. crispatus* LMG P-9437 and *B. breve* LMG S-28195 and we studied the effect of specific combinations of these two strains with other probiotic strains already available on the market, in order to improve their immunomodulatory action. In the case of myasthenia gravis, we obtained a significant improvement of the symptoms of the disease when given the combination of bifidobacteria while treatment with the lactobacilli was not equally efficient. In the second model of autoimmune disease used we have not observed statistically significant differences between lactobacilli and bifidobacteria, the clinical manifestations are in fact significantly improved both in the case of administration of the lactobacilli which in the case of administration of the strains of bifidobacteria, although this second treatment proved slightly more efficient. These data could be a promising new therapeutic approach for the treatment of autoimmune diseases, the future development will

be in fact the translation of these experiments in the *in vivo* context, by testing strains in patients with these diseases.

Finally, strains were screened also for a food application. The activity was carried out in collaboration with the Institute of Geology and Agro-Food Engineering of the Catholic University of Piacenza and it had the aim of investigate the possibility of producing spray-dried probiotic formulations to be used together with commercial ice-cream bases. A commercial cream base was selected for the study and, according to its composition and literature, two different formulations were investigated (one with maltodextrins, widely used in the food industry and spray-drying processes and one with inulin, a prebiotic fibre with both beneficial effects for humans and probiotics preservation) and used for encapsulation the strain *L. paracasei* LMG S-27487. The results show that the maltodextrins formulation could not be used for probiotics encapsulation (null process yield), while inulin formulation gave a lower yield (51 %). A 82 % death cell occurred during drying, but the cells survived in the ice-cream making process. So, these results represent an important starting point for the development of encapsulated multiple bioactive ingredients containing probiotics or other functional compounds.

Summarizing, in this study I showed that the adoption of logical criteria for the *in vitro* selection of probiotic bacteria can result in the isolation of strains able to exert different effects mainly on human health. However, as reported in the recent literature, it appears unlikely that a single probiotic bacteria strain is able to exert the most different functions but, on the contrary, it is more likely that these effects are strain specific as observed in my study.

Supplementary material

Supplementary material

Table 1 shows, for each bacterial strain, the values of the count (expressed in CFU/ml) obtained in the presence of pepsin in the culture medium, respectively, after 0, 1, 90, 180 minutes of incubation of the strains at 37 ° C in anaerobic conditions.

	T0	T1	T90	T180
<i>L. paracasei</i> LMG S-27487	2,4 X 10 ⁹	4,8 X 10 ⁸	3 X 10 ⁵	< 1 X 10 ³
<i>L. paracasei</i> CBA-L101	3,4 X 10 ⁹	1,0 X 10 ⁹	< 1 X 10 ⁵	< 1 X 10 ³
<i>L. paracasei</i> CBA-L145	6,8 X 10 ⁹	1,2 X 10 ⁹	< 1 X 10 ⁵	< 1 X 10 ³
<i>L. paracasei</i> CBA-L144	1,9 X 10 ¹⁰	3,0 X 10 ⁹	4,8 X 10 ⁵	2 X 10 ²
<i>L. plantarum</i> CBA L137	2,2 X 10 ⁹	4,7 X 10 ⁸	< 1 X 10 ⁵	< 1 X 10 ³
<i>L. plantarum</i> CBA-L138	1,6 X 10 ⁸	1,5 X 10 ⁷	< 1 X 10 ⁵	< 1 X 10 ³
<i>L. plantarum</i> CBA-L150	7,6 X 10 ⁹	2,2 X 10 ⁹	1,4 X 10 ⁴	2 X 10 ²
<i>L. rhamnosus</i> CBA-L92	2,9 X 10 ⁹	1,1 X 10 ⁹	6 X 10 ⁵	< 1 X 10 ³
<i>L. rhamnosus</i> CBA-L142	5 X 10 ⁹	1,1 X 10 ⁹	1 X 10 ⁵	3 X 10 ³
<i>L. rhamnosus</i> CBA-L140	5,3 X 10 ⁹	1,5 X 10 ⁹	6 X 10 ⁵	< 1 X 10 ³
<i>L. rhamnosus</i> CBA-L143	1,1 X 10 ¹⁰	3,3 X 10 ⁹	1,9 X 10 ⁷	7 X 10 ⁵
<i>L. acidophilus</i> CBA-L63	1,2 X 10 ⁹	2,9 X 10 ⁸	4 X 10 ⁵	3,7 X 10 ⁴
<i>L. rhamnosus</i> GG	7,3 X 10 ⁹	1,3 X 10 ⁹	2 X 10 ⁵	< 1 X 10 ³
<i>L. gasseri</i> CBA-L72	4,1 X 10 ⁹	9,8 X 10 ⁸	8,6 X 10 ⁷	2,3 X 10 ⁴
<i>L. fermentum</i> CBA-L146	5,4 X 10 ⁹	2 X 10 ⁹	9 X 10 ⁶	2 X 10 ³
<i>L. fermentum</i> CBA-L147	4 X 10 ⁹	4,4 X 10 ⁸	3 X 10 ³	< 1 X 10 ²
<i>L. mucosae</i> CBA-L148	1,6 X 10 ⁹	4,1 X 10 ⁸	8 X 10 ³	< 1 X 10 ²
<i>L. mucosae</i> CBA-L149	3,7 X 10 ⁹	8,2 X 10 ⁸	9,1 X 10 ³	< 1 X 10 ²
<i>L. vaginalis</i> CBA-L88	3,6 X 10 ⁹	8,9 X 10 ⁸	1,3 X 10 ⁵	2,0 X 10 ⁴

Table 1: ability of the bacterial strains to grow in the presence of pepsin in the culture medium

The results show how the CFU/ml after only one minute of contact of the bacterial culture with the solution of pepsin are, at worst, less of a decimal logarithm compared to those obtained at time zero; the bacterial vitality was also reduced drastically after 90 minutes and then decreased again until reaching a value in the order of $10^2 - 10^3$ CFU/ml after 180 minutes. Only the strains *L. rhamnosus* CBA-L143, *L. acidophilus* CBA-L63, *L. gasseri* CBA-L72 and *L. vaginalis* CBA-L88 after being exposed for 180 minutes with the pepsin solution still have a bacterial vitality in the order of $10^4 - 10^5$ CFU/ml.

This assay despite the fact that it is easy to perform and cheap, it presents some defects because it does not reflect the ideal conditions such as the residence time in the gastrointestinal tract of the bacterial strains which varies depending on the subject. Just because these in-vitro tests as described by Papadimitriou *et al.*, 2015 expose the bacterial strain to too strong stress conditions that do not represent what happens in the gastrointestinal tract during food consumption, I re tested some bacterial strains with another method proposed by Corcoran *et al.*, 2005 that used less restrictive conditions in fact it expected a pepsin concentration decrease and a reduction of the contact time of the strains with it. Using the latter method, I could prove an increase in the percentage of bifidobacteria and lactobacilli able to survive.

References

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