UNIVERSITY OF MODENA AND REGGIO EMILIA

**PhD School in AgriFood Sciences,
Technologies and Biotechnologies**

Development of probiotic strains for specific targets and strategies for maintenance of viability

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XXV Cycle

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Final Thesis Defense 2014

Il processo di una scoperta
scientifica è, in effetti,
un continuo conflitto di meraviglie.

(*Albert Einstein*)

ACKNOWLEDGMENTS

My special gratitude goes first to my supervisor, Professor Maddalena Rossi, for her vast assistance in preparing and editing my work, continuous encouragement, direction and reassurance. Her meticulous comments and suggestions were an enormous help to me.

I would particulary like to express my gratitude to Dr. Giovanni Mogna of Probiotical SpA for his scientific and financial support.

I would also like to thank Professor Andrea Pulvirenti, who was very supportive expecially during the last year.

Finally, I would like to thank my husband for the great support and my family who always encouraged me and wished success for this study.

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AAS	Atomic absorption spectrophotometry
BSH	Bile salt Hydrolase
CFU	Colony forming units
CPA	Cryo-protective agent
DMEM	Dulbecco modified minimal essential medium
DNA	Deoxyribonucleic acid
F6PPK	Fructose-6-phosphoketolase
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
HPLC	High performance liquid cromatography
LAB	Lactic acid bacteria
MRS	De Man, Rogosa and Sharpe
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
QPS	Qualified presumption of Safety
RDA	Recommended Daily Allowance
RSM	Reconstituted skim milk
SCFA	Short chain fatty acid
SD	Standard deviation
SM	Semidefined medium
SRB	Sulphate-reducing bacteria
TEER	Transepithelial electrical resistance
Tg’	Glass transition temperature
TPY	Tryptone Phytone Yeast broth

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INTRODUCTION

1. Intestinal microbiota

The mammalian gut is one of the most densely populated microbial ecosystems on Earth. In particular, the colon is populated by a complex community composed largely of anaerobic bacteria, whose cell numbers exceed 10^{11} per gram of intestinal content. This vast microbiota fulfills its energy needs using, through hydrolytic and fermentative pathways, the substrates left undigested and unabsorbed in the small intestine. Oligo- and polysaccharides, proteins and peptides are broken down by bacterial enzymes into their oligomeric and/or monomeric components. Then, sugars and amino acids are fermented, yielding organic acids (such as lactic, acetic, propionic, and butyric acids), branched chain fatty acids (such as isobutyric, isovaleric, and 2-methylbutyric acids), H_2, CO_2, ammonia, amines and several other end-products. Short chain fatty acids (SCFA) are, from a nutritional point of view, the major fermentation products. Butyrate is of particular importance because it is the main energy source for the epithelial cells that line the colon. SCFA affect the metabolism, growth, and differentiation of colonocytes, influence the hepatic control of lipids and carbohydrates, and provide muscle, kidney, heart, and brain with energy (O'Keefe 2008).

The gut microbiota exherts metabolic, trophic and protective functions (Bourlioux, 2014). Trophic functions include control of cell proliferation and stability of the immune system. Metabolic functions are based on the fermentation of available substrates which have escaped digestion in the upper GIT and, to a lesser extent, sloughed off intestinal epithelial cells. In the mutualistic relationship between humans and commensal intestinal bacteria, the bacterial metabolism and the cross-talk between bacteria and epithelial cells have a major impact on the nutrition and the overall health status of the host. Microorganisms are provided with a broad spectrum of fermentable compounds and a temperature controlled anoxic environment, while the host gains the protection against infections, the modulation of the immune system,

and the supply of carbon, energy, vitamins, and bacterial-activated dietary metabolites (Leser and Mølbak 2009, O'Keefe *et al*. 2009).

1.1. Development of a mature microbiota

In the womb, the human fetus inhabits a sterile environment. From a microbiological point of view, the most significant factor associated with the birth of a human being is that the environment is transformed from one free of microbes to a microbe-dominated situation. On delivery, the neonate encounters, for the first time, a wide range of microbes from a variety of sources (Morelli, 2008).

The main sources of microbes that colonize a neonate are:

- Vagina, gastrointestinal tract (GIT), skin, oral cavity, and respiratory tract of the mother;
- Skin, respiratory tract, and oral cavity of other individuals present at the delivery;
- Instruments and equipment used during delivery;
- The immediate environment.

Within a very short time of delivery, microbes are detectable on those surfaces of the baby that are exposed to the external environment, i.e. the eyes, skin, respiratory tract, genito-urinary system, GIT, and oral cavity. What is surprising, however, is that, despite the neonate's exposure to such a variety of microbes, only a limited number of species are able to permanently colonize the different body sites, and each site harbors a microbial community comprised of certain characteristic species, i.e. the microbes display "tissue tropism" (Palmer *et al.*, 2007). The organisms found at a particular site constitute what is known as the indigenous (or "normal") microbiota of that

site, and they are termed "autochthonous" (i.e. native to the place where they are found) species.

Processes leading to the development of a stable intestinal microbiota are very complex and imply a succession of different microbial genera from childhood to adulthood (Mackie *et al.*, 1999). In the womb the fetus is in a sterile environment, therefore from the moment of the birth, he is exposed to microbes from the mother and from the surrounding.

During conventional birth, microorganisms from the vagina and faeces of the mother contaminate the newborn infant. Furthermore, after birth, environmental microorganisms and oral and skin mother's bacteria are transferred to the baby through nursing and physical contact and after few days the colonization of the GIT occurs (Simon and Gorbach, 1984). First microorganisms able to colonize GIT, after few hours from the birth, are aerobic and facultatively anaerobic bacteria, such as *Escherichia coli* and *Streptococcus* spp. (10^8-10^{10} cfu/ g of faeces). These relatively nutritionally undemanding bacteria lower the redox potential in the intestine, creating a highly reduced environment which allows the development of the strictly anaerobic bacteria that will later dominate the colon (Favier *et al.*, 2002).

The type of delivery, dietary constituents, and gestational age influence the colonization pattern. Dependent on the type of feeding regime of the early life, the development of microbiota can undergo enormous variability: the brest-fed infants have a preponderance of bifidobacteria, which easily outcompete other genera, while formula-fed infants have a more complex microbiota which resembles the adult gut in that bacteroides, clostridia, bifidobacteria, lattobacilli, Gram positive cocci, coliforms and other groups are all represented in fairly equal proportions (Edwards *et al.*, 2002).

The relatively simple microbiota in the brest-fed infant remains until dietary supplementation occurs. Upon introduction of other foods to the diet of the brest-fed infants the differences between brest-fed and formula-fed infants disappear. There is then a transitional period, until the second year of

life, when the intestinal microbiota evolves to resemble that of the adult (Stark *et al.*, 1982).

Initial bacterial colonization is very relevant for determining the composition of permanent microbiota in adults. The first bacteria colonizing GIT can modulate genes expression in intestinal epithelium cells, thus creating a favorable habitat for themselves and hindering the growth of other bacteria which subsequently enter in the ecosystem (Mackie *et al.*, 1999). The composition of the bacterial microbiota in the large intestine and faeces of different age groups may differ. The most prevalent bacteria in the faeces of infants and adults are obligate anaerobes, while facultative anaerobes are generally expected to account for less 10^3 of anaerobe numbers. In adults, the microbiota of large intestine is more complex than that of of children. The stools of adults have a low redox potential, a neutral or slightly alkaline pH, a typical odour and colour, and they contain relatively large amounts of putrefactive products, such as ammonia, amines, phenols, and degraded bile acids (Hopkins *et al.,* 2002; Hebuterne,2003).

1.2. The human-microbe symbiosis

The concept of "normal microbiota" was born at the end of XIX century, when physiologists and microbiologists discovered that in addition to pathogenic bacteria, many other non pathogenic microorganisms lived normally in human body. Microorganisms occurring on the human body surface, on mucous and in feces of healthy individuals were defined as "normal microbiota". This concept was later replaced with "indigenous microbiota", indicating a community establishing symbiotic relationship with the host (Savage, 1977). The term "indigenous microbiota" encompasses all of the bacteria, archaea, viruses, fungi, and protoctists present on the body's surfaces. However, most studies have investigated only the bacterial component of the microbiota, and little is known about the identity of the other types of microbes present at any body site.

Colonization by microbes of the newborn at birth marks the beginning of a life-long human–microbe symbiosis. Symbiosis means "living together", and the term can be applied to any association between two or more organisms. When the species comprising a symbiosis differ in size, the larger member is known as the host while the smaller is termed a "symbiont". At least three types of symbiotic associations are recognized:

(1) mutualism – when both members of the association benefit;
(2) commensalism – when one member benefits while the other is unaffected;
(3) parasitism – when one member suffers at the expense of the other.

During the course of his/her lifetime, a human being will experience all three types of symbiotic relationships with various members of their indigenous microbiota. One microorganism is able to colonize an epithelial surface if has two important characteristics: adhesion and survival capacity (Savage *et al.,* 1979). Adhesion is performed through membrane specialization (such as proteins and fimbriae), enzymes and toxins production, and metabolic advantage in absorption of available nutrients. All these factors are instruments used by microorganisms to overcome the host defense mechanisms. The bacterial adhesion is influenced by the so-colled "tropism", through which bacteria bind to specific receptors of the host cells in specific anatomic sites offering environmental conditions more favorable for the colonization of certain groups of microorganisms (Chisari *et al.*, 1992).

Tropism is influenced by physiologic characteristics of the host, such as temperature, resistance to bile acids, diet composition, drugs and integrators use, and food quantity stagnating in the colonization site. One of the many remarkable features of the microbiota of a particular body site is that its composition among different humans beings, is similar despite the huge variations in the climate to which they are exposed, the diet that they consume, the clothes that they wear, the hygiene measures that they practice, and the lifestyle that they adopt (Wilson, 2005). This implies that humankind has co-evolved with some of the microbial life forms that are present on Earth to form a symbiosis that is usually of mutual benefit to the participants.

1.3. Nature and distribution of the microbial communities

The indigenous microbiota of humans consists of a large number of microbial communities, each with a composition that is characteristic of a particular body site (Hill,1990). In an adult human, microbes outnumber mammalian cells by a factor of ten – the average individual consists of 10^{13} mammalian cells and 10^{14} microbial cells. With few exceptions (the eyes, stomach, duodenum, and certain skin regions), the communities consist of large numbers of microbes and a large variety of species. Most of the microbes inhabiting humans are present in the GIT – the relative numbers in the GIT, in the mouth, in the vagina, and on the skin being approximately 1.000.000 : 100 : 10 : 1, respectively (Orrhage *et al.*, 2000). Although most body surfaces that are exposed to the external environment are colonized by microbes, some are not (e.g. the lungs), and the population density of those sites that are colonized varies markedly from site to site. Hence, the colon, the oral cavity, and the vagina are densely populated by microbes, while much smaller numbers are present on the eyes, in the stomach, in the duodenum, and in the urethra. Even within an organ system, the density of colonization, as well as the community composition, can vary enormously from site to site (Skinner *et al.*, 1974). For example, in the respiratory tract the upper regions are more densely populated than the lower regions – in fact, the *bronchi* and *alveoli* are usually sterile.

The complexity of the microbial community depends on the particular body site – only organisms able to grow under the conditions prevailing at the site being able to survive and grow there. The number of species present in the community can range from one or two at sites in which the environment is not conducive to microbial growth or survival (e.g. the conjunctiva) to more than 800 at sites such as the colon, which offers a microbe-hospitable environment containing a wide variety of nutrients (Eckburg *et al.*, 2005).

The gastrointestinal tract (GIT), together with the accessory digestive organs (i.e. teeth, tongue, salivary glands, liver, gallbladder, and pancreas), constitutes the digestive system, whose function is to break down dietary

constituents into small molecules and then to absorb these for subsequent distribution throughout the body. The GIT consists of several anatomically and functionally distinct regions – the oral cavity, the pharynx (which is also part of the respiratory tract), oesophagus, stomach, small intestine (duodenum, jejunum, and ileum) and the large intestine (cecum, colon, and rectum). Essentially, however, it can be considered to be a continuous tube extending from the mouth to the anus.

The environmental determinants within each region of the GIT are very different, and each region, therefore, has a distinctive microbiota (Gill *et al.*, 2006).

Nowhere is this more apparent than in the oral cavity, where the complex anatomy, the presence of shedding and non-shedding surfaces, and the existence of large mechanical forces combine to provide a group of very different habitats. In contrast, each of the other regions of the GIT has a simpler structure, is not subjected to large mechanical forces, and does not contain non-shedding surfaces; consequently, each has a lower habitat diversity than that of the oral cavity (Dewhirst *et al.*, 2010).

The GIT has a number of distinct regions, each housing a characteristic microbial community or communities. In the upper GIT (oral cavity, pharynx, and esophagus), the resident microbiota is associated with surfaces, and because materials (food, secretions, etc.) passe rapidly through these regions, microbial communities cannot become established in their lumens. As the passage of material becomes slower in the lower regions of the GIT, there is an opportunity for communities to develop within the lumen as well as on the mucosal surface – in the distal ileum, cecum, colon, and rectum such communities are substantial (Nava *et al.*, 2011).

Very few studies have investigated the esophageal microbiota. Culture-dependent studies of this region have shown that it is dominated by staphylococci, lactobacilli, and *Corynebacterium* spp., while a culture-independent study found streptococci and Gram-negative anaerobes (*Prevotella* spp. and *Veillonella* spp.) to be dominant (Pei *et al.*, 2004).

Because of its low pH, the stomach is a hostile environment for a wide range of organisms. The vast numbers of bacteria (in saliva and food) that are continually entering the stomach make difficult to distinguish between autochthonous species and transients (Hao *et al.*, 2004). Organisms detected in the lumen are mainly acid-tolerant species of streptococci and lactobacilli together with staphylococci, *Neisseria* spp., and various anaerobes. These organisms are also present in the mucosa-associated community which, in addition, often contains the important pathogen *Helicobacter pylori* (Zilberstein *et al.*, 2007).

The environments within the duodenum and jejunum are also largely inimical to many microbes because of the low pH, the presence of bile and other antimicrobial compounds, and the rapid transit of material. Consequently, the mucosa and the lumen of both of these regions have sparse microbiotas consisting mainly of acid-tolerant streptococci and lactobacilli.

In the ileum, expecially the terminal region, conditions are less hostile to microbes, and the microbiota within the lumen and on the mucosa is more substantial. Streptococci, enterococci, and coliforms are the dominant organisms in the lumen, but the microbiota of the mucosa is very different and consists of high proportions of anaerobes including *Bacteroides* spp., *Clostridium* spp., and *Bifidobacterium* spp. (Booijink *et al.*, 2007).

The cecum has a lower pH and a higher content of easily fermentable compounds than the more distal regions of the GIT and, consequently, it harbors microbial communities very different from those in the rest of the large intestine (Nava *et al.*, 2011). The lumen is dominated by facultative organisms (mainly enterobacteriaceae and lactobacilli), although substantial proportions of anaerobes (*Bacteroides* spp. and *Clostridium* spp.) are also present. Little is known about the mucosal microbiota, although it appears to be dominated by *Bacteroides* spp.

1.4. The colonic microbiota

The colon is colonized by a very large and diverse microbial population. Up to 80% of the organisms present have not yet been grown in the laboratory, and many of these are novel phylotypes (Flint *et al.*, 2007;Hold *et al.*, 2002). Culture-dependent studies of the fecal microbiota have revealed that obligate anaerobes are 1000-fold greater in number than facultative organisms – the predominant genera being *Bacteroides*, *Eubacterium* (and related genera), *Bifidobacterium*, and *Clostridium* (Duncan *et al.*, 2007). However, the use of culture-independent approaches has given a different picture and has revolutionized the understanding of the microbial communities that are present in the colon (Dethlefsen *et al.*, 2006; Flint, 2006). Moreover, molecular profiling has shown that the communities associated with the colonic mucosa differ from those in feces, although the exact nature of such differences remains to be established (Wang et al., 2003).

The human gastrointestinal tract of humans contains all three domains of life (bacteria, archaea, and eukarya), but it is predominated by bacteria belonging to *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Eckburg et al. 2005). *Firmicutes* are by far the most abundant and diverse group, and include the *Clostridia* and *Bacilli* class; *Bacteroidetes* are also present in high numbers. The dominant organisms in feces are members of the *Firmicutes* (mainly the *Clostridium coccoides–Eubacterium rectale* group, and *Clostridium leptum*) and, to a less extent, the *Bacteroidetes*. Other groups present in substantial proportions include *Bifidobacterium* spp. and members of the *Atopobium* cluster (Zoetendal et al., 2004). Both culture-dependent and culture-independent studies of the microbiota of the rectal mucosa have shown that members of the *Bacteroidetes* are the dominant organisms (Hayashi *et al.*, 2005).

More than 500 different bacterial species may be present in the normal large intestine microbiota, although the exact number and the variability among individuals remains an area of investigation (Mai, 2004). The information on microbial diversity within this complex community experienced a great implementation in recent years thanks to the molecular

techniques based on 16S rDNA sequencing (Dethlefsen *et al.*, 2006; Flint, 2006). This analysis indicated that more than 75% of the phylotypes found in the large intestine does not match known and cultivable microbial species (Flint *et al.,* 2007; Hold *et al.,* 2002).

The large colonic microbiota is rather stable (Ramakrishna, 2007; Abell, 2007). Significant variations are detected among the transient or aerobic species such as lactobacilli and *Escherichia coli*. The native species belong to the genera *Bacteroides*, *Bifidobacterium*, *Faecalibacterium*, *Prevotella*, *Rikenella*, *Acetivibrio*, *Butyricicoccus*, *Oscillibacter*, *Dorea*, *Anaerostipes*, *Coprococcus*, *Peptostreptococcus*, *Porphyromonas*, *Eubacterium*, and *Ruminococcus* (Fig. 1: Nava *et al.*, 2011). Other genera are present in much smaller concentrations, namely *Fusobacterium*, *Streptococcus*, *Lactobacillus*, *Veillonella*, *Megasphaera*, *Propionibacterium*, and *Enterobacteriaceae*, the latter being a large family of bacteria that includes many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella*.

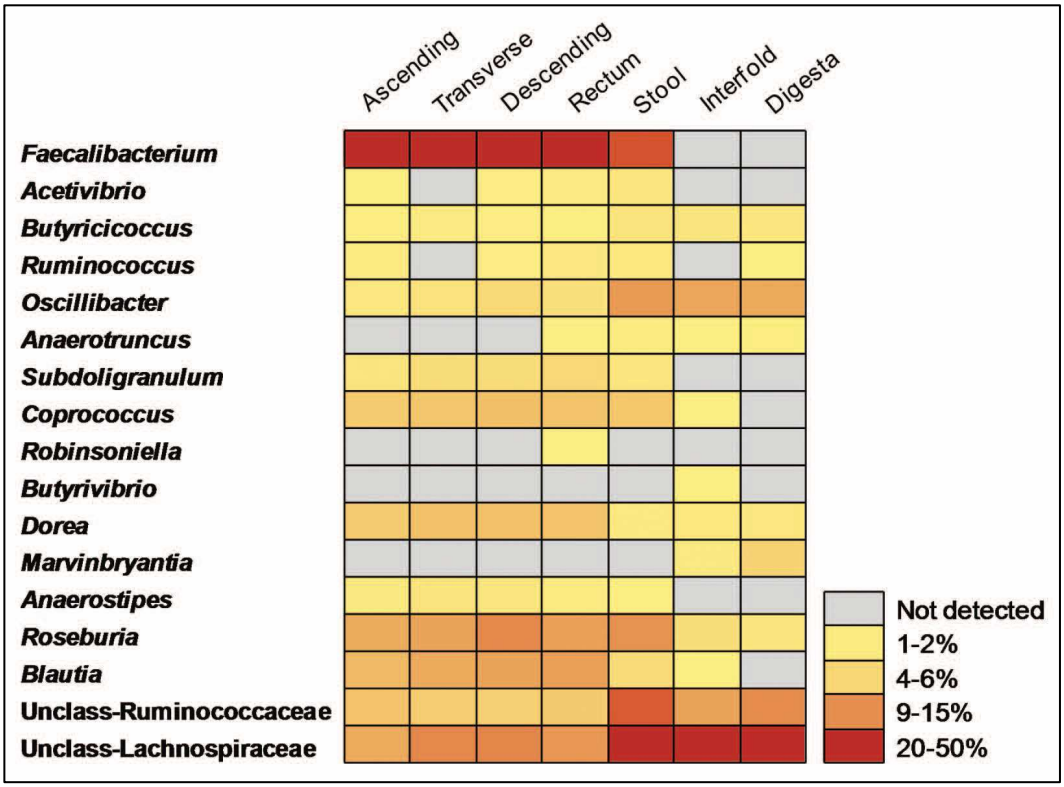


Figure 1. Common and specific phylogenetic genera of autochthonous bacteria associated to the colonic mucosa of both mouse and human (Nava et al., 2011).

This complex ecosystem presents an enormous capacity of fermenting both carbohydrate and proteins (Manning *et al.*, 2004). Virtually all carbohydrates reaching the colon can be fermented by a mixture of species depending on the composition of the monomeric units, the degree of polymerization and branching, and solubility. In general, the short-chain carbohydrates are fermented faster than long-chain ones.

The ascending colon is the most suitable segment for the growth of microorganisms thanks to both the slight acidity of the intestinal content (pH between 5.4 and 5.9) and the large amount of fermentable substrates. In the transverse and descending colon the pH gradually increases and the carbohydrates availability is lower than in the ascending portion, therefore the replication of bacterial cells is slowed (Manning *et al.*, 2004; Blaut *et al.,* 2007).

1.5. The metabolism of intestinal microbiota

The intestinal microbiota and the host have very strong metabolic interactions. Such interactions should not be seen only from a mere nutritional point of view, as they are able to significantly affect the host's health. In fact, many enzymes of microbial origin can catalyze chemical transformations of either food components or substances produced by the host, altering the biochemical, physiological, and immunological features of the intestine (Backhed *et al.*, 2005).

While the transit of residual foodstuffs through the stomach and small intestine is probably too rapid for the microbiota to exert a significant impact, it slows markedly in the colon. The average transit time is about 70 hours; as such, colonic microorganisms have ample opportunity to degrade available substrates, derived both from diet or by endogenous secretions.Fermentations by GIT bacteria consists of a series of energy yielding reactions that do not use oxygen in the respiratory chains. The fermentation process in the large gut is influenced by a variety of physical, chemical, biological, and environmental factors, such as the amount of substrate available for fermentation, the colonic

transit time, the physical form of the substrate, the pH of gut content, the composition of gut microbiota with respect to species diversity and relative numbers of different types of bacteria, competitive and cooperative interactions between bacteria, the antibiotic therapy, the availability of inorganic electron acceptors (Fleming *et al,* 1983; Koecher *et al.*, 2014)

The carbon and nitrogen sources available in the colon, which the microbiota is adapted to use, are constituted by carbohydrates, proteins and peptides that are not hydrolyzed by the host digestive enzymes and reach the colon in an undigested or only partially digested form. The degradation of these substances is carried out by all the metabolic activities of the bacterial community and has the effect of establishing interactions of synergy or nutritional interdependence, as well as competition between different microbial groups.

Major substrates for saccharolytic metabolism available for the colonic fermentation are starches resistant to the action of pancreatic amylases, than degraded by bacterial enzymes, and dietary fibers like pectins and xylans; in lower percentage, also olisaccharides and sugar or sugar alcohols can be fermented (Salminen et al., 1998). Due to absorption in the small intestine, the colon is an environment pretty deprived of simple sugars such as mono- and disaccharides. However, oligo- and polysaccharides that are resistant to hydrolysis in the small intestine reach the colon at pretty high concentrations. They either have a dietary origin (e.g., fibers, resistant starch, pectins) or are produced by the host (e.g., glycoproteins and glycoconjugates). In the colon most of them are depolymerised and extensively fermented by bacteria (Salminen *et al.*, 1998).

The ability to use and metabolize these insoluble fibers is associated to the presence of bacterial groups called "primary degraders", as in the case of degradation of cellulose (Robert *et al.*, 2003) and mucin (Derrien *et al.,* 2004). Recent *in vitro* studies have shown that the use of a certain substrate is highly species-specific and that the reasons for this specialization may lie in the need fora proper binding, degradation, and uptake of the substrate (McWilliam Leitch *et al.,* 2007).

These microbial consortia, including bacteria involved either in the primary or in the secondary degradation, play a key role in the complex microbial ecology of the intestine. It has been shown that the metabolic capabilities may differ between bacteria adhering or not adhering to the food residues, thus highlighting the crucial role of the ability to develop biofilms (MacFarlane *et al.,* 2006). The fermentation of carbohydrates is essential for the microecology and the physiology of the large intestine. It produces lactic acid and SCFA (short chain fatty acids, namely acetic, propionic, and butyric acid) which are absorbed by the host or used by other microbial components (Cummings *et al.*, 1991; Hoverstad *et al.,* 1984a; Hoverstad *et al.*, 1984b; Ruppin *et al.,* 1980). Therefore the hydrolysis of complex carbohydrates creates the opportunity not only for competition but also for cooperation by metabolic cross-feeding.

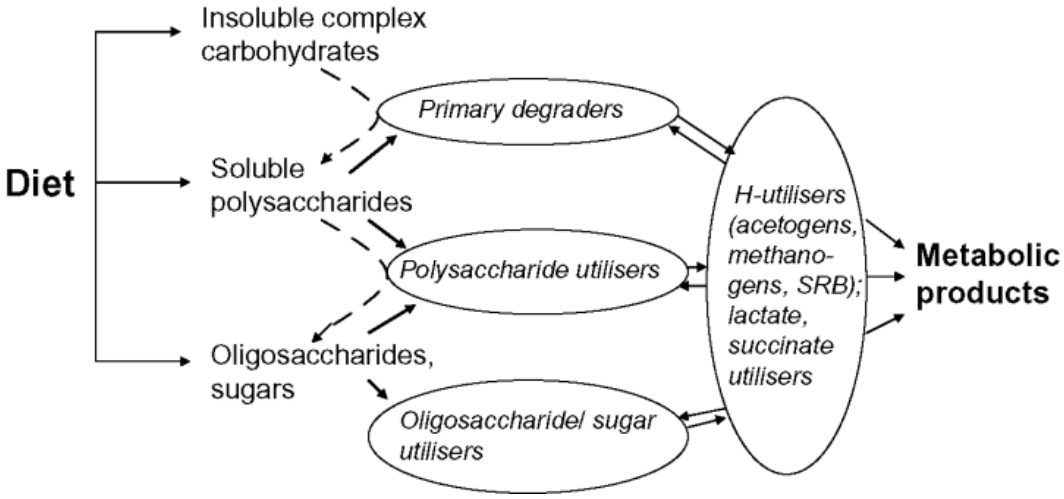


Figure 2. The role of cross –feeding interactions in the conversion of non-digestible carbohydrates by the anaerobic microbial community of the large intestine. SRB, sulphate-reducing bacteria (Flint *et al.*, 2007).

These interactions include hydrogen consumption and uptake of fermentation and hydrolysis products such as lactate, succinate and oligosaccharides coming from the hydrolysis of complex polymers. Some studies have shown that in the intestinal ecosystem it is possible the

conversion of lactate into both butyrate and propionate (Bourriaud *et al.,* 2005; Morrison *et al.,* 2006). Therefore the competition between different bacterial groups able to use lactate, including sulphate-reducing bacteria (SRB), may be an important factor in determining the balance of fermentation products.

Carbohydrates metabolism is quantitatively more important in the ascending tract of the colon, where there is the highest availability of fermentable substrates. The production of organic acids causes a significant decrease in the pH of this portion of intestine, thus hindering the growth of putrefactive and potentially pathogenic proteolytic bacteria. In addition, the short-chain fatty acids (SCFA) exert a stimulatory effect on the uptake of water and electrolytes by the colon mucosa and inhibit the proliferation of neoplastic colonic epithelial cells (Livesey *et al*., 1995; Kim *et al.,* 1994).

Most of the colonic bacterial groups (*Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, and *Clostridium*) are saccharolytic, even if there are significant differences in the degradation ability and in the kinetics of growth. In fact, the glycosyl hydrolase activities responsible for polysaccharides metabolism are variously distributed among the different groups. While bifidobacteria are able to grow at high rates in presence of elevated concentrations of carbohydrates, *Bacteroides* are competitive when the carbon sources are limited and usually grow at a lower rate (MacFarlane *et al.,* 1995; Salminen *et al.,* 1998).

In the colon proteins and peptides may result from food, bacterial lysis, or endogenous sources (Chacko *et al*., 1988; Cummings *et al*., 1997). They come from food residues, enzymes produced by the host or by the intestinal microbiota, mucus glycoproteins, and proteins released after the epithelial cells desquamation or the lysis of intestinal bacteria. The colonic microbial community actually represents one of the ecosystems with the highest proteolytic activity known to date. The proteases produced by certain microbial groups hydrolyze proteins to amino acids and simple peptides that represent an important nitrogen source for the entire microbiota, including obligate saccharolytic bacteria. Amino acids and peptides can also be used as

carbon and energy sources by bacteria strictly dependent on amino acids metabolism or able to ferment both amino acids and carbohydrates (Macfarlane *et al.,* 1995a; MacFarlane *et al.,* 1995b; Salminen, 1998).

Clostridium, *Bacteroides*, *Eubacterium*, and *Peptococcus* are the major proteolytic bacteria in the gut (Macfarlane *et al.*, 1988). The secretion of proteases by these microbial groups occurs in response to nutrients limitation and takes place primarily in the descending colon. During the transit along the large intestine the concentration of fermentable carbohydrates is gradually reduced till depletion, causing the shift from a fermentative metabolism of carbohydrates to a proteolytic putrefaction, as highlighted by the progressive increase of pH. Amino acids are catabolized through reactions of deamination, hydroxylation, dehydroxylation, desaturation, and decarboxylation, with production of harmful molecules for the host health, such as ammonia, biogenic amines, and phenols (MacFarlane *et al.*, 1998). The ammonia passes through the gut mucosa and exerts a toxic effect on the brain, while biogenic amines, especially when a liver failure compromises their detoxification, could be significantly harmful as they affect the circulation, muscle activity, and mediate mutagenic and carcinogenic activities. Phenols, primarily skatole (3-methylindole) and indole, are strongly carcinogenic (Karlin *et al.,* 1985).

One of the most important functions of the intestinal microbiota is the so-called "barrier effect", such that a new microorganism introduced through food in a normal host is eliminated in a few days (Ducluzeau *et al.,* 1970), thus preventing its potential for proliferation. This competitive exclusion hinders many allochthonous microorganisms to colonize the digestive system and, if this is the case, to exert any pathogenic activity. This effect contributes to maintain the concentration of potentially pathogenic groups such as some species of *Clostridium* or most *Enterococcus* below a certain threshold. The impairment of the barrier effect can lead to more serious intestinal disorders, often caused by the growth of opportunistic agents such as *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio cholerae*, and *Clostridium difficile* (Gilmor *et al.,* 2003; Lievin *et al.,* 2006).

2. Probiotics

In the light of the impact of intestinal microbiota on health and wellness of the host, different strategies aimed to modulate its composition were defined, namely the control of external factors such as diet or drugs intake, or the oral administration of specific alive microbial bacteria called "probiotics". The term "probiotic" was coined in 1965 by Lilly and Stillwell who first described some substances produced by a microorganism able to stimulate the growth of other bacteria, which were called "probiotics" as opposed to the term "antibiotic" (Lilly *et al.,* 1965). The word "probiotic"is translated from the Greek meaning "for life". Another definition was given by Parker (1974): "Organisms and substances which contribute to to intestinal microbial balance". However, this was subsequently refined by Fuller (1989) as: "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". This latter version is the most widely used definition and has gained widespread scientific accepatability. A probiotic would therefore incorporate living microorganisms, seen as beneficial for gut health, into diet.

Probiotics are supplements or food ingredients that contain live bacteria, which are beneficial to health (Salminen *et al.*, 1998). According to another definition, a probiotic is "a live microbial food supplement that beneficially affects the host animal by improving the microbial balance" and it could be used in fermented dairy products, cheeses and food supplements (Gorbach, 1996). A more detailed definition regards a probiotic as a "live, non-pathogenic microorganism capable of reaching the bowel alive and vital and to benefit the host, whenever administered in adequate quantities" (Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, 2002). Recent evidence, emerged from effectiveness studies carried out on humans, has suggested the use of the name "biotherapeutic agents" for probiotic strains capable of effectively and innovatively contributing to medical therapies.

Probiotics have a long history, being the first records about the intake of bacterial drinks by humans over 2000 years old. The first bacterium used as probiotic was *Lactobacillus delbrueckii* subsp. *bulgaricus*, present in fermented milk traditionally widespread in Bulgarian population and isolated at the beginning of the last century by Ilya Ilyich Metchnikoff; the Nobel Prize awarded was the first to assume that lactic acid bacteria could improve human health. The gut is an obvious target for the development and testing of functional foods because it acts as an interface between diet and the metabolic pathways of human health. A later study, carried out in 1964 by American researchers on the Masai people, who consume large amounts of fermented milk, have extremely low cholesterol levels, and are rarely affected by coronary diseases, opened up the way for the era of probiotics as they are currently being used (Mann *et al.,* 1964). Subsequent research has been directed towards the use of intestinal isolates of bacteria as probiotics (Fernandes *et al.*, 1987). Over the years, many species of microorganisms have been used. They mainly consist of lactic acid producing bacteria (lattobacilli, streptococci, enterococci, lactococci, bifidobacteria), but also *Bacillus* spp. and fungi such as *Saccharomyces* spp. and *Aspergillus* spp.

Probiotics for human use are currently applied in various sectors by the dairy, pharmaceutical, and the functional foods industries. Many different species and strains are used, but the most common are (Goossens *et al.*, 2003):

***Lactobacillus* spp.**: *L. acidophilus, L. crispatus, L. johnsonii, L. jensenii, L. gasseri, L. casei, L. paracasei*, *L. delbrueckii* subsp. *bulgaricus, L. reuteri, L. brevis, L. cellobiosus, L. curvatus, L. salivarius, L. fermentum, L. plantarum,L. rhamnosus* (including *Lactobacillus* GG);

***Bifidobacterium* spp.**: *B. bifidum*, *B. longum*, *B. adolescentis*, *B. breve*, *B. infantis, B. lactis*;

***Streptococcus* spp. and other lactic acid bacteria (LAB)**: *Streptococcus salivarius* subsp. *thermophilus*, *Streptococcus diacetylactis, Lactococcus lactis* subsp. *cremoris.*

All the above mentioned species can claim a so-called Qualified Presumption of Safety (QPS), similar in concept and purpose to the GRAS (Generally Recognized As Safe) definition used in the USA, and jointly

defined by the European Food Safety Authority (EFSA) and the former DG SANCO Scientific Committees on Food, Animal Nutrition and Plants.

Not all probiotics are intended for human consumption and generally the criteria for use are based upon two elements:

- the host species;
- the desired effects.

The use of probiotics in animal nutrition has essentially reproductive purposes as their intake determines an increase in speed of development, a lower incidence of subclinical infections, an increase in quantity (poultry) of eggs and milk (cows) produced, as well as an improved ability to metabolize the feed thanks to the enzymes produced by the probiotics (Gaggìa *et al.,* 2010).

2.1. Claimed beneficial properties of probiotics

So far, it was shown that the indigenous microbiota is host-specific and location specific, very complex in composition and that it has beneficial properties to the host. However, it is not precisely known what species of microorganisms play the principal part in these beneficial properties. For the humans, it has been suggested that specific microbial strains could play an important role in:

✓ **Formation and reconstruction of a well-balanced indigenous intestinal microbiota**

Several studies have assessed the effectiveness of lactobacilli in the prevention and treatment of antibiotic-associated diarrhea (AAD) and confirmed the true effectiveness of many strains, especially in the adult population (Johnston *et al.,* 2011; Kale-Pradhan *et al.*, 2010). Benefits have been proven also in the prevention of acute childhood diarrhea, the reduction of both severity and duration of Rotavirus-associated acute diarrhea, and the reduction in the risk

of contracting traveler's diarrhea (Saavedra *et al.,* 1994; Katelaris *et al*., 1995).

✓ **Metabolising lactose and hence reducing lactose intolerance**

The most extensively studied probiotic applications involve the intestinal tract (Aureli *et al.,* 2011; Marteau *et al.,* 1993). In lactose intolerant people the ability of yogurt and probiotics to improve lactose digestion has been demonstrated, mainly by delaying gastric emptying and intestinal transit, thus causing slower delivery of lactose to the intestine, optimizing the action of residual β-galactosidase in the small bowel, and decreasing the osmotic load of lactose (Gilliland, 1990; Montalto *et al.,* 2006). The bacterial β-galactosidase activity of probiotics is a responsible for improving lactose digestion (de Vrese *et al.,* 2001).

✓ **Formation and reconstruction of a well-balanced indigenous respiratory microbiota.**

The actual mechanism of action of probiotics is subject to constant, increasingly in-depth studies, as regards beneficial effects manifested at gut level and in other parts of the body as well, including the respiratory tract, for which evidence of effectiveness is constantly increasing (Pregliasco *et al.,* 2008). More recent efficacy trials have shown the potential beneficial effect of probiotics on respiratory system, specifically as regards the prevention and reduction of duration and severity of acute respiratory infections (ARI) with a concomitant increase in IgA secreting cells in the bronchial mucosa (Pregliasco *et al.,* 2008; Perdigon *et al.,* 1999). It has been reported that probiotics can reduce also the incidence and severity of respiratory infections in children (Hatakka *et al.,* 2001).

✓ **Non-specific interactions with the immune systems**

Further evidences suggest that the use of probiotics can benefit patients affected by type 2 diabetes mellitus, self-immune diseases and chronic inflammatory diseases, such as rheumatoid arthritis and bowel inflammatory diseases, and also enhance immunization against flu through increased IgG

and IgA serum levels (Bunout *et al.*, 2002; Hatakka *et al.*, 2003; Roberfroid, 2000a). A positive impact has also been seen in the treatment of atopic diseases, with a reduction of the SCORAD (SCORing Atopic Dermatitis) in children and adults affected by atopic dermatitis or atopic eczema, asthma, food intolerances, and allergies (Kalliomaki *et al.,* 2001; Weston *et al.,* 2005; Prescott *et al.,* 2005; Yoo *et al.*, 2007; Bjorksten, 2005; Majamaa *et al.,* 1997; Drago *et al.*, 2011). Many healthy properties are most likely mediated by the qualitative and quantitative modulation of the mucosal immune response mediated by some specific probiotic strains (Matsuzaki *et al.*, 2000). Innate immune response cells, such as macrophages and dendritic cells, express membrane receptors, particularly from the Toll-like family (TLR2 and TLR4), that are capable of recognizing molecular patterns found on the surface of many bacterial species that are part of the intestinal microbiota, including probiotics (Travassos *et al.*, 2004). More specifically, activation through different TLRs leads to distinct cytokine production and secretion by dendritic cells (DCs), thus inducing different functional states in the dendritic cells themselves and polarizing T-cell responses (Qi et al., 2003; Mohamadzadeh et al., 2005). Such recognition typically results in a considerable increase in specific inflammatory response mediators, such as interleukins and immunoglobulins, particularly IgAs, thus improving mucosal immune response (Tlaskalova-Hogenova *et al.*, 2004).

- ✓ **Lowering the serum cholesterol level**

Mediated either by Bile Salts Hydrolase (BSH) enzymes secreted by some probiotics, able to deconjugate bile acids in the gut lumen, or by direct cholesterol absorption by bacterial membranes of certain probiotics (Patel *et al.*, 2010). Both mechanisms of action lead to a decrease in plasmatic concentration of cholesterol, particularly beneficial in case of mild to severe hypercholesterolemia (Bordoni *et al.*, 2013).

- ✓ **Inhibition of nitrate reduction**

The organic acids produced by bifidobacteria and lactobacilli are able to inhibit the development of many intestinal nitrate-reducing bacteria

(*Escherichia coli*, *Proteus, Pseudomonas*) (Klatte *et al.*, 2011). The nitrite ions oxidize hemoglobin ferrous ions to ferric ions, transforming it into methaemoglobin, typically unable to bind oxygen. This is a particular risk in infants, for which it is important to avoid taking water rich in nitrates (Hugo *et al.,* 2006; Weng *et al.,* 2011).

✓ **Synthesis of group B vitamins**

Certain strains, especially belonging to *Bifidobacterium* genus and *Lactobacillus reuteri* species, have the capability to synthesize water-soluble vitamins such as those included in the B group, namely folates (vitamin B9), biotin (vitamin B7), cobalamin (vitamin B12) and/or riboflavin (vitamin B2) (Pompei *et al.*, 2007a; Pompei *et al.*, 2007b; Strozzi *et al.,* 2008; LeBlanc *et al.*, 2011).

✓ **Treatment and/or prevention of vaginal infections caused by *Candida***

Certain probiotic strains, usually belonging to the species *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus gasseri*, and *Lactobacillus rhamnosus* are able to colonize the vaginal mucosa and secrete specific bacteriocins able to directly and specifically antagonize different species of *Candida*, including *Candida albicans*, therefore solving a vulvovaginal candidiasis (VVC) in a very short time and preventing possible recurrences (Coudeyras *et al.*, 2008; Rönnqvist *et al.,* 2007).

✓ **Reduction of oxidative stress**

Specific microorganisms are able to produce antioxidant molecules, primarily glutathione and superoxide dismutase (SOD), which could be absorbed through the intestinal wall and exert a systemic positive impact on the average level of oxidative stress (Hathout *et al.*, 2011; Martarelli *et al.*, 2011).

✓ **Treatment of gaseous colics in infants**

Many newborns and infants suffer from gaseous colics, often responsible for prolonged crying spells and severe discomfort. Differences in gut microbiota

were found among colicky and non-colicky infants: colicky infants were less frequently colonized by *Lactobacillus* spp., and more frequently by anaerobic Gram-negative bacteria. Many of these, namely belonging to the genera *Enterobacter* and *Klebsiella*, have been isolated from colicky infants and they are thought to be directly involved in colics onset and maintenance. In fact, gas forming coliforms may be involved in determining colonic fermentation and consequently excessive intraintestinal air load, aerophagia and pain, characteristic symptoms of colic crying. Only very specific probiotic microorganisms, many of them isolated from infants, have the ability to directly inhibit such Gram-negative bacteria, thus offering a significant relief to colicky babies (Savino *et al.*, 2004; Savino *et al.,* 2010; Savino *et al.*, 2011).
Other innovative benefit that could be attributed to probiotics is the improvement of the absorption of calcium and hence the inhibition of the decalcification of the bones in elderly people**.**

2.2. Requirements for a probiotic strain

A microorganism whose purpose is the use as probiotic in food supplements or pharmaceutical products should have certain physiological features related not only to the beneficial effects exerted on the host but also to practical aspects involved in a perspective of large-scale production.

It is therefore desirable that the microorganism:

▪ is able to reach the gut in a viable condition and at a high concentration. For this purpose the large-scale production yield is mandatory, in order to supplement a high number of viable cells per dose (the minimum daily dose is generally regarded as 1 billion viable cells/day). Stability during and after the lyophilization process or other methods of drying is another major parameter;

▪ is able to survive passage through the stomach and upper intestine. In fact,

as probiotics have to pass through the gastro-duodenal tract before arriving to the gut, an unavoidable reduction in the number of viable cells occurs. In order to be effective and confer health benefits to the host, probiotics must be present in sufficient amount to impact the colon microenvironment. This feature is strongly strain-dependent, even if it could be considered that on average 10 to 25% of the intaken cells actually survive and reach the gut, thus exerting their probiotic benefits (Del Piano *et al.*, 2006);

▪ is able to persists in the gut for a long time or at least during the whole administration period of the probiotic supplement. The ability to adhere to the intestinal epithelium promotes long-term colonization, thus allowing a reduction of the daily dose administered. A permanent colonization has the advantage of prolonging the beneficial effects even after treatment discontinuation;

▪ does not bear any trait of antibiotic resistance. In fact, even if it is true that an antibiotic resistance allows the survival of the probiotic strain even during a concomitant antibiotic therapy, on the other side it very likely poses the risk of horizontal transmission (e.g., by conjugation) of the resistance genes to pathogens present in the bowel. The European Food Safety Authority (EFSA) has defined the resistance thresholds for 9 to 10 antibiotic molecules, depending on the probiotic species. Some resistances are considered to be intrinsic, therefore with totally negligible risk of transmission (e.g., vancomycin resistance in most species of heterofermentative lactobacilli) (EFSA, 2008);

▪ is exhaustively characterized from a genotypic and phenotypic point of view. It is generally believed that probiotics should have a human origin, even if it has been quite well demonstrated that bacteria coming from plant or animal sources, such as corn silages, chickens or pigs faecal material, may exert beneficial and innovative effects as well, while being as safe as strains of human origin;

▪ has no adverse effect. Probiotics typically belong to species claiming a Qualified Presumption of Safety (QPS), don't produce gases such as H_2, CH_4 or H_2S, and are free of potentially toxic or harmful enzymes.

2.3. Applications of probiotics

The number of food and other dietary adjuncts products containing live *Bifidobacterium* and *Lactobacillus* bacteria have significantly increased over the last 20 years, due in part to the beneficial effects these probiotic organisms are believed to provide (Laroia and Martin, 1990). It is estimated that well over 10 million people in Europe regularly (each day) consume probiotics. The European market value is in excess of one billion euros per annum. Many different products exist and new developments are continuing at a rapid pace. Probiotics are marketed as health or functional foods, and claims are made for health benefits resulting from changes induced in the gut microbiota.

There is a need for consumers to be provided with an independent assessment of physiological, microbial, and safety aspects of these live microbial products and their ability to improve health. The approach of using diet to prophylactically manage the gut microbiota is both user friendly and attractive to the consumer.

The market progression in recent years has also been rapid and there are now many examples of foods that contain probiotics:

- *Live yoghurts:* also called bio, active, bifidus. Manufactures like Nestlè, St Ivel, Danone, Onken, Vifit, introduced probiotic bacteria into yoghurt. These bioyoghurts differ from conventional yoghurts in that other strains are added, or used in manufacture, in addition to the standard starter species (*Lactobacillus delbreuckii* subsp.*bulgaricus* and *Streptococcus Thermophilus).*

- *Fermented dairy drinks:* these liquid products contain high microbial numbers (up to 10^9 per ml). Examples are Yacoult *(Lactobacillus casei* Shirota), Danone's Actimel (*(Lactobacillus casei* Immunitass), and Nestle's LC1Go (*Lactobacillus johnsonii).*
- *Freee-dried supplements:* freeze-drying of microbial cultures allows extremely high numbers to be incorporated into capsules, sachets and tablets, in formulations for nutraceutical applications (Hilliam, 2003).
- *Cheese*: examples of probiotic cheese are Inner Gut (Anchor), stracchino probiotico con *Lactobacillus acidophilus,* (Stella Bianca), gorgonzola Gran Cuore (Santi).
- *Fruit juices:* a widely used form is Proviva which contains *Lactobacillus plantarum* 299V.

In Table 1 is presented a list of some bacterial species used as probiotic cultures in food products.

Table 1. Bacterial species primarily used as probiotic cultures (Krishnakumar and Gordon, 2001)

Species	**Strains**
Lactobacillus acidophilus	*La2, La5(also known as La1), Johnsonii(La1; also known as Lj1), NCFM, DDS-1, SBT-2062*
L.bulgaricus	*Lb12*
L.lactis	*La1*
L.plantarum	*299v, Lp01*
L.rhamnosus	*GG, GR-1, 271, LB21*
L.reuteri	*SD2112(also known as MM2)*
L.casei	*Shirota, Immunitass, 744, 01*
L.fermentum	*RC-14*
Bifidobacterium longum	*BB536, SBT-2928*
B.breve	*Yakult*
B.bifidum	*Bb-12*
B.lactis	*Bb-02*

2.4. Lactic acid bacteria (LAB)

Lactic acid bacteria are a group of Gram-positive and catalase negative bacteria united by a constellation of morphological, metabolic and physiological characteristics. They are non–sporing, carbohydrate–fermeting lactic acid producers and acid tolerant of non-aerobic habitat.
Typically they are non-motile and do not reduce nitrate (Bergey's Manual).

They are subdivided into four genera: *Streptococcus, Leuconostoc, Pediococcus and Lactobacillus*. Recent taxonomic revisions suggest that lactic acid bacteria group could be comprised within the genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus.* Originally, Bifidobacteria were included in the genus *Lactobacillus* and the organism was referred to as *Lactobacillus bifidus*. The term lactic acid bacteria was used synonymously with "milk souring organisms".

Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic acid producing bacteria of other habitats was recognized (Axelsson, 1993). Lactic acid bacteria are generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also members of the normal microbiota of the mouth, intestine, and vagina of mammals. The genera that, in most respects, fit the general description of the typical lactic acid bacteria are (as they appear in the latest Bergey's Manual from 1986) *Aerococcus*, *Streptococcus, Leuconostoc, Pediococcus,* and *Lactobacillus*.The genera *Leuconostoc, Pediococcus* and *Lactobacillus* have largely remained unchanged, but some rod-shaped lactic acid bacteria, previously included in *Lactobacillus,* is now forming the genus *Carnobacterium* (Collins *et al.* 1987).

2.4.1. The genus *Lactobacillus*

The genus *Lacobacillus* is by far the largest of the genera included in lactic acid bacteria. It is very heterogeneous, encompassing species with a large variety of phenotypic, biochemical and physiological properties. The heterogeneity is reflected by the range of mol % G+C of the DNA of species included in the genus (32-53%, that is twice the span usually accepted for a single genus). Lactobacilli are Gram-positive, non-spore forming, rods or coccobacilli with a G+C content of DNA usually > 50% (Bergey's Manual).

They are widly spread in nature, strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic, and have complex nutritional requirements. Lactobacilli are found where carbohydrates are available, and thus in a variety of habitats including mucosal membranes of humans and animals, mainly in oral cavity, intestine and vagina, plant material, and fermented foods (Hammes *et al.*1991).

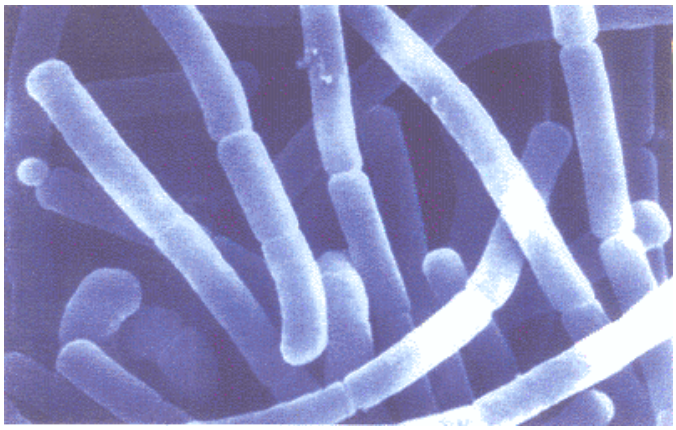


Figure 3. Scanning electron microscope (SEM) image of *Lactobacillus acidophilus*

The research of Orla-Jensen's in 1919 was directed to identify these bacteria, in particular those found in the Danish dairy cheese; at that time he recognized 10 species. In the latest 9th edition of the Bergey's Manual, 44 species have been recognized. The latest grouping of lactobacilli by Kandler

and Weiss (1986) relies on biochemical and physiological criteria and neglects classical criteria of Orla-Jense such as morphology and growth temperature, since many of the recently described species did not fit into the traditional classification scheme.

Lactobacilli have been later divided in three metabolic groups (Hammes and Vogel , 1995):

Group A: *Obligatory homofermentative lactobacilli*: Hexoses are fermented to lactic acid; pentose and gluconate are not fermented.

Group B: *Facultatively heterofermentative lactobacilli*: Not only hexoses are fermented to lactic acid; pentoses and often gluconate can be fermented, since this group possess the enzymes aldolase and phosphoketolase.

Group C: *Obligatory heterofermentative lactobacilli*: Hexoses are fermented to lactate, acetic acid and CO_2 in equimolar amounts; pentose may be fermented.

2.4.2. The genus *Streptococcus*

The genus *Streptococcus* contains about 60 species and a number of them is known for their pathogenicity. The species *S. thermophilus,* however, consists of GRAS/QPS microorganisms, very essential in the dairy manufacturing which makes it one of the most commercially important LAB. It is Gram-positive, catalase negative, non-motile, non-spore-forming and homofermentative. Cells are ovoidal or spheroidal shape with a diameter of 0.5-1 μm, tipycally organized in chains. In association with *L. delbrueckii* subsp. *bulgaricus,* it is used as starter culture for the production of yoghurts. Actually 40 species of *Streptococcus* are known, mostly located on human and animal mucosal surfaces; other have been found in water, vegetables, foods, and soil (Bergey's Manual).

S. thermophilus strains have the maximum of growth at temperatures between 37°C and 45°C, and have a lactose permease, called lacS (Poolman *et*

al., 1989), which allows the disaccharide to enter into the cell. The sugar inside the cell is then cleaved by a β-galactosidase (lacZ) (Schroeder *et al.,* 1991) into glucose and galactose; the latter is then usually excreted from the cell at the same speed as lactose entry and metabolism. It was also observed that the synthesis of β-galactosidase is inhibited by the presence of glucose.

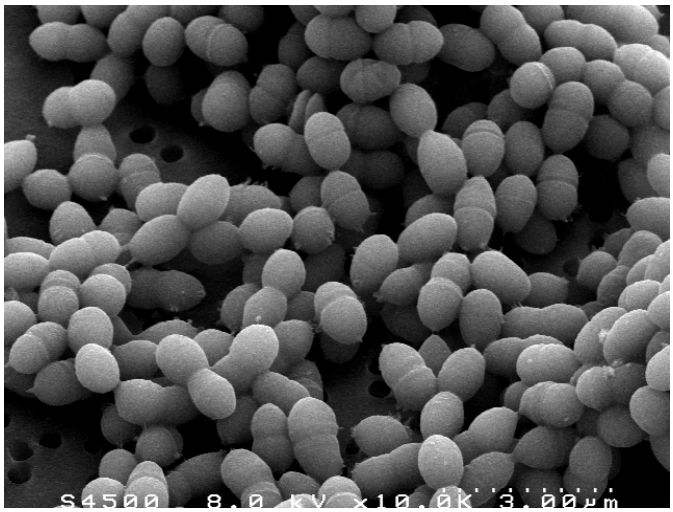


Figure 4. Scanning electron microscope (SEM) image of *Streptococcus thermophilus*.

S. thermophilus is able to produce diacetyl from citrate and to hydrolyze casein thanks to a specific protease. This species is widely used for the preparation of dairy products, in particular in the manufacture of yoghurt and soft cheeses such as mozzarella, stracchino and crescenza.

2.4.3. The genus *Lactococcus*

Lactic acid bacteria growing at the temperature of 30°C belong to this species, with obligatory homofermentative metabolism and L-lactic acid as principal product of fermentation (Teuber *et al.* 1993).

The *Lactococcus* genus includes few species, three of which have a particular interest in industrial application:

L. lactis susbspecies *lactis;*

L. lactis susbspecies *cremoris;*

L. lactis susbspecies *diacetylactis lactis.*

The first two can grow in a 2% NaCl substrate; *L. diacetylactis* subspecies may use citric acid to produce diacetyle.

2.5. Bifidobacteria

2.5.1. History

In 1899, Henry Tissier, a French pediatrician at the Pasteur Institute in Paris, isolated a bacterium characterised by a Y-shaped morphology ("bifid") in the intestinal microbiota of breast-fed infants and named it "bifidus". The scientist observed that these bifidobacteria were dominant in the gut microbiota of healthy babies. He thus noticed that when the bacteria were present in an infant's intestinal microbiota, the baby suffered less from gastrointestinal disorders; he therefore recommended treating infants suffering from diarrhea with these bacteria.

Later Orla-Jensen found that *Bacillus bifidus* also produced lactic acid, then he classified it in the family of Lactobacteriaceae with the name *Lactobacillus bifidus.* Although in 1924 Orla-Jensen suggested that the genus *Bifidobacterium* should be regarded as a separate taxon, the name *Lb. bifidus*, solely based on morphological elements and on analysis of fermentation products, remained until the ‘70s. In fact, the assignment of a certain taxonomic location to *B. bifidus* was made particularly complicated by its high polymorphism.

As soon as the cultivation in complex culture mediums was made possible, Dehnert instituted a classification of bifidobacteria in 5 groups on the basis of morphological elements and the ability to ferment 24 sugars (Dehnert, 1957). Reuter in 1963 used fermentative and serological properties as a classification criterion, thus identifying 8 species, all isolated from humans: *B. bifidus* var. a and b, *B. infantis*, *B. parvulorum* var. a and b, *B. breve* var. a and b, *B. liberorum*, *B. lactensis*, *B. adolescentis* var. a and b, *B. longum* var. a and b.

The studies took advantage from the identification of a specific fermentative pathway typical of bifidobacteria (Scardovi *et al.,* 1965; De Vries *et al.,* 1967), whose key enzyme turned out to be the fructose-6-phosphate phosphoketolase. Such enzymatic activity was found in cell extracts and allowed the attribution to the genus *Bifidobacterium*. A much more significant taxonomic criterion derived from DNA/DNA hybridization studies (Scardovi *et al*., 1970; Scardovi *et al.,* 1971) which quantitatively established the homology degree between the species.

The result of the reclassification on the basis of these last criteria led to recognition of 25 species belonging to the genus *Bifidobacterium*, mentioned in Bergey's Manual of Systematic Bacteriology. Five more recently identified species were added: *B. gallinarum* (Watabe *et al*., 1983), *B. gallicum* (Lauer, 1990), *B. ruminantium* (Biavati *et al.,* 1991a), *B. merycicum* (Biavati *et al.,* 1991a), and *B. saeculare* (Biavati *et al.,* 1991b). They occur in animal and human habitats, in particular they have been isolated from faeces, rumen of cattle, sewage, human vagina, dental caries, and honey bee intestine.

2.5.2. Morphologic, physiological and metabolic features

Bifidobacteria are Gram-positive polymorphic branched rods that occur singly, in chains or clumps. The rod shape is maintained only in optimal nutritional conditions, such as those characterizing their natural habitat, while in laboratory cultures they assume forms with irregular bulges, bumps, and ramifications. This pleomorphism, although not fully elucidated, has been attributed to their peculiar nutritional requirements probably due to a cell wall composition more complex than other bacterial genera. They are non-spore forming, non-motile, and non-filamentous. They are anaerobic and chemoorganotrophs, having a fermentative type of metabolism. They produce acid, but not gas from a variety of carbohydrates. They are catalase negative, with some exceptions (*Bifidobacterium indicum* and *Bifidobacterium asteroids*

when grown in presence of air). Their genome G+C content varies from 42 to 67 mol% (Biavati and Mattarelli, 2001).

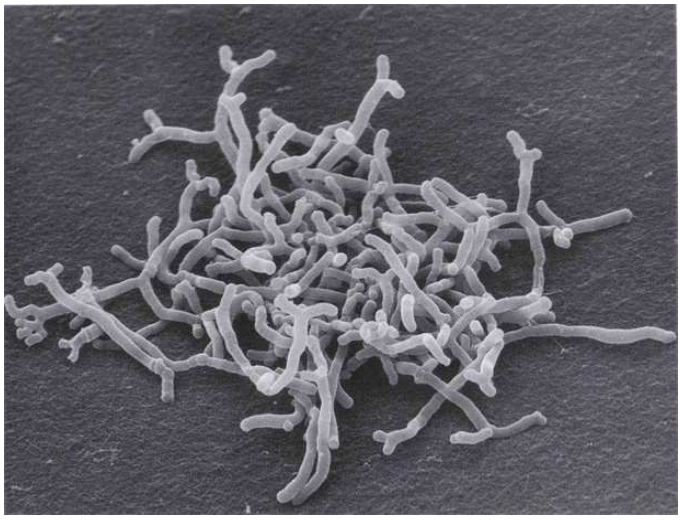


Figure 5. Scanning electron microscope image of *Bifidobacterium longum*. http://jpkc.njau.edu.cn/spwswx/imgbank/tuku/Bifidobacterium%20longum.jpg

According to the Taxonomic Outline of Prokaryotes (Garrity *et. al.*, 2004) the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class A*ctinobacteri*a, subclass A*ctinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. To date, 29 species are included in the genus *Bifidobacterium*. Bifidobacteria degrade hexoses through a peculiar metabolic pathway, the so-called bifidus shunt, the fructose-6-phosphate pathway, the key enzyme of which is the fructose-6-phosphoketolase. This enzyme was considered a taxonomic character for the identification of the genus level (Biavati and Mattarelli, 2001), but, due to the reclassification of *Bifidobacterium* species into new genera, it can be considered a taxonomic marker for the family *Bifidobacteriaceae.*

The optimal conditions for growth of bifidobacteria are temperatures between 36 and 38°C, a pH between 6 and 7 (pH values lower than 5 hamper the growth), strict anaerobiosis, and low redox potential of the medium. As a matter of fact, different strains present a variable tolerance to oxygen, thanks to a weak catalase activity.

The *Bifidobacterium* genus has rather heterogeneous nutritional requirements. Many species use only ammonium salts as a nitrogen source (Hassinen *et al.,* 1951), while others need organic compounds (Matteuzzi *et al.,* 1978), such as urea if presenting urease activity (Crociani *et al.,* 1982). The nitrogen taken is then converted by microorganisms in various amino acids such as alanine, valine and aspartic acid. The need for vitamins is variable within the genus (Trovatelli *et al.*, 1978). The addition of riboflavin to the culture medium is generally required, while other water-soluble vitamins such as thiamine, folic acid, nicotinic acid, pyridoxine and vitamin B12 are generally synthesized by the cell (Deguchi *et al.,* 1985). Studies concerning the need for metal ions have been conducted on *B. bifidum* by Bezkorovainy and Topouzian (Bezkorovainy *et al.*, 1983; Bezkorovainy *et al.*, 1986) who clarified some aspects related to metabolism and mechanisms of uptake of Fe^{2+}, indicating the presence of electrogenic pumps dependent on proton gradients.

Bifidobacteria are saccharolytic organisms, although some strains have a weak proteolytic activity (Borisova *et al.,* 1973), and use as a carbon source monosaccharides such as glucose, galactose, and fructose, disaccharides such as lactose and sucrose, trisaccharides such as raffinose (a selective medium for bifidobacteria is Raffinose-*Bifidobacterium* agar, RB) (Hartemink *et al.,* 1996), and some complex polysaccharides able to reach the colon indigested, such as mucopolysaccharides produced by mammals, glycoproteins of meat, plant oligosaccharides and polysaccharides such as fructans, pectins, hemicelluloses and cellulose. The complex polysaccharides are degraded by extracellular or surface enzymes and the hydrolysis products are then transported intracellularly to be used in fermentation processes. Consistently, it has been shown that *B. bifidum* and some strains isolated from the oral cavity and from rumen have an extracellular α-1,6-glucosidase responsible for the degradation of dextran. Oligosaccharides and disaccharides, once transported inside the bacterial cell, are hydrolyzed to monosaccharides by specific intracellular glycosidases, some of which have been well characterized (von Nicolai *et al.*, 1981; Katayama *et al.*, 2005; Sakai *et al.*, 1987).

The fermentation of glucose is carried out through the shunt of fructose 6-phosphate (Scardovi *et al.*, 1965; De Vries *et al.,* 1967), typical of bifidobacteria, while the glycolytic pathway and the hexose monophosphate shunt are missing. The process starts with the conversion of glucose 6-phosphate into fructose 6-phosphate by hexokinase and glucose 6-phosphate isomerase. The fructose 6-phosphate is then hydrolyzed to acetyl-phosphate and eritrose 4-phosphate by the fructose-6-phosphoketolase, the most important enzyme of this fermentation process. Acetyl-phosphate is then converted into acetate by the enzyme acetate kinase. The eritrose 4-phosphate and the fructose 6-phosphate are converted into xylulose 5-phosphate through the subsequent action of transaldolase and transketolase. The phosphorolytic cleavage of xylulose 5-phosphate leads to the formation of glyceraldehyde 3-phosphate and acetyl-phosphate. From glyceraldehyde 3-phosphate pyruvic acid is obtained, which is then reduced to L-(+)-lactic acid. The fermentation of glucose mainly produces acetic acid and L-(+)-lactic acid according to the theoretical ratio 1.5/1.0. Some deviations from this fermentative ratio could occur and they are attributed to the phosphoroclastic cleavage of at least part of pyruvic acid into formic and acetic acids, and the reduction of the latter to ethanol (De Vries *et al.,* 1968).

3. Functional foods

Modern food science has moved beyond the classical concepts, mainly based on avoiding nutrient deficiencies and on the adequacy of the common dietary habits, and embraced the concept of "positive" or "optimal" diet. Research is currently focused on identifying the biologically active food components with the potential of optimizing the physical and mental wellbeing and reduce the risk of contracting diseases. It was found that many traditional foods, including fruits, vegetables, soybeans, integral cereals and milk, contain components potentially beneficial to health. In addition to these, new foods are being developed with the aim of strengthening or

incorporating such benefits as useful components for their positive effects on health or their favourable physiological effects.

The concept of functional foods originated in Japan. In the 1980s, health authorities of this country recognised the need to improve the quality of life in the light of the increased life expectancy of an expanding number of elderly people. It was therefore introduced the concept of foods specifically developed to promote health or reduce the risk of diseases. Moreover, thanks to a better understanding of the interactions between genes and nutrition (Kok, 1999), the individual biochemical and nutritional needs began to receive greater emphasis.

According to Roberfroid (Roberfroid, 2000b) the term "functional food" should not be intended as a well-defined and characterized entity but rather as a real concept.

Indeed, functional foods can have multiple definitions, as many as the number of authors referring to them:

1. foods that may provide health benefits beyond basic nutrition (IFIC Foundation, 1995);
2. foods or food products marketed with the message of the health benefit (Riemersma, 1996);
3. food and drink products derived from naturally occurring substances consumed as part of the daily diet and possessing particular physiological benefits when ingested (Hillian, 1995);
4. food derived from naturally occurring substances, which can and should be consumed as part of the daily diet and which serves to regulate or otherwise affect a particular body process when ingested (Smith *et al.*, 1996);
5. food similar in appearance to conventional food, which is consumed as part of the usual diet and has demonstrated physiological benefits and/or reduces the risk of chronic disease beyond basic nutritional functions (Health Canada, 1997);

6. food that encompasses potentially helpful products, including any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrient contained (Food and Nutrition Board, 1994). Whatever definition is considered, some important and common features are apparent.

A functional food should:

- ✓ be a conventional food;
- ✓ be intaken as a normal part of the diet;
- ✓ consist of natural and not synthetic ingredients;
- ✓ have positive effects on one or more physiological functions;
- ✓ help preserve and/or improve health and overall wellbeing and/or reduce the risk of onset of diseases directly related to diet;
- ✓ have supportive scientific evidence.

Following the growing interest in the concept of functional foods and health claims, the European Union has created a Concerted Action of the European Commission on Functional Food Science in Europe (FUFOSE). This program was coordinated with the aim of establishing and developing an approach scientifically based on the evidence required to support the ideation and development of food products that may exert beneficial effects on a specific biological function, thus improving the health and general wellbeing of the subject and/or reducing the risk of disease.

The report takes the position that functional foods should be in the form of normal foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. A functional food can be a natural whole food, a food to which a component has been added, or a food from which a component has been removed by technological or biotechnological means. It could also be a food in which the nature of one or more components has been modified, or a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities. A functional food may be addressed to the general population or

to particular groups, which may be defined, for example, by age or by genetic constitution. The EU Concerted Action supports the development of two types of health claims relevant to functional foods, which must always be valid in the context of the whole diet and must relate to the amounts of foods normally consumed.

The European Directive 1924/2006/CE defined the criteria for health claims assessment, classification, plausibility and acceptability in the light of the supportive efficacy studies available. It is actually a real turning point in the world of food supplements and functional foods with the main aim of consumer protection through strict labelling rules.

Pursuant to this Directive, health claims should be classified as:

- ✓ Type A (Art. 13.1 and 13.5): "Enhanced function" claims that refer to specific physiological, psychological functions and biological activities beyond their established role in growth, development and other normal functions of the body. This type of claim makes no reference to a disease or a pathological state (e.g., certain non-digestible oligosaccharides improve the growth of a specific bacterial group in the gut; caffeine can improve cognitive performance).
- ✓ Type B (Art. 14): "Reduction of risk of disease" claims that relate to the consumption of a food or food component that might help reduce the risk of a specific disease or condition because of specific nutrients or non-nutrients contained (e.g., folates can reduce a woman's risk of giving birth to a child with neural tube defects, and sufficient calcium intake may help reduce the risk of osteoporosis in later life).

Currently the European Union still lacks a specific legislation addressed to this category of foods (Roberfroid, 2002b), with only a few countries having a well-defined regulatory framework regarding definition, labelling and marketing of functional foods. In Japan, for example, such foods are recognised and marketed with the code FOSHU (Food for Specific Health Use) and functional properties have been proved by scientific intervention investigations in humans.

AIM OF THE THESIS

Reseach and innovation in the field of probiotics is often related to the identification of novel targets, in order to develop properly selected strains that can act for specific pathological problems, preventing, retarding, or curing.

In this perspective, two defined targets have been identified as research topics:

1. probiotics as vehicle of minerals;
2. oxalate degrading probiotics.

A still partially unexplored topic is the study of probiotic bacteria that can work as vehicle of minerals. The development of an optimized process to internalize selenium and zinc in *Lactobacillus buchneri* Lb26 (DSM 16341) and *Bifidobacterium lactis* Bb1 (DSM 17850), respectively, has been faced in this study. These two strains are the outcome of a previous screening that demonstrated that the capability to bind these metals is a special feature of few strains (Leonardi *et al.*, 2013). Following the selection of specific strains, it is of pivotal importance to develop a process that allows to obtain the highest yield of biomass, with the highest amount of internalized mineral.

The exploitation of probiotics as mineral vehicles is an hypothesis that has never been validated. In order to demonstrate that the organically bound metals associated to probiotic biomass present a higher bioavailability, a biological method to evaluate the bioavailability of Se and Zn contained in the two probiotic strains was assessed. In particular, a cell culture model with CaCo-2 cells in bicameral chambers was developed and the comparison of the transport across the cell culture among minerals-enriched probiotics and inorganic and organic forms of the two minerals was performed.

The potential role of probiotics in the degradation activity of oxalate has recently become a major research interest. However, only few data on the specific field are still currently available and functional studies on the

effectiveness of lactic acid bacteria in reducing hyperoxaluria are not in an advanced stage. In this study, the *in vitro* oxalate-degrading activity of *Lactobacillus* and *Bifidobacterium* probiotic strains was evaluated. More in detail, each probiotic was grown in a medium containing oxalate as a sole carbon source and, after incubation in appropriate conditions, oxalate was quantified by reverse-phase HPLC.

Once the selection of probiotic strains has been carried out taking into account their specific physiological features and their interaction with the host, it is important to care of the process that leads to the production of high amount of the selected strains and that allows to maintain as long a possible their viability. In fact, from an industrial point of view, specifically selected probiotics become industrial products if two conditions are fulfilled:

1. it is possible to obtain the products at high yields during the fermentation process;
2. the product can be processed in order to maintain its viability as long as possible.

The industrial use of lactic acid bacteria as probiotic cultures depends on the preservation techniques employed, which are required to guarantee stable cultures in terms of viability and functional activity. Freeze-drying is the classical method used to produce dry bacterial powders, because drying takes place at low temperatures, thus reducing heat degradation. However, freeze-drying may cause different types of cellular damages that may affect the viability and activity of the microorganisms. Cell injury can be attributed mainly to changes in the physical state of the membrane lipids or in the structure of sensitive proteins. Different compounds such as sugars, antioxidant substances, aminoacids, and proteinaceous products have been tested in the past to improve the survival of bacteria.

In this perspective, the influence af a variety of cryoprotectant media on the freeze-drying process of a *Lactobacillus acidophilus* NCIMB 701748 has been investigated. This strain was selected on the basis of its quite high

sensitivity to freezing. Specifically, the objective was to evaluate the protective effect of trehalose, sucrose and maltodextrin with or without glycerol, using two different freeze-drying cycles.

As a whole, the project has been organized describing the three major topics as single projects:

1. probiotics as vehicle of minerals;
2. oxalate degrading probiotics;
3. influence of cryoprotectants on the viability of freeze-dryed *Lactobacillus acidophilus*.

PROBIOTICS AS VEHICLE OF MINERALS

1. Background

The internalization of minerals in order to achieve an improved bioavailability after oral administration can represent an innovative application of probiotics. It has been demonstrated that the form of the trace elements affects the intake efficiency in animals. Several studies reported that certain organic compounds of trace elements (including iron, zinc, magnesium, and selenium) are more bioavailable than the inorganic forms, possibly because the mechanisms for absorption adapted to these kinds of nutrients during species evolution (Teucher *et al.*,2004; Coudray *et al.*,2005; Mazo *et al.*, 2007; Peters *et al.*, 2008; Navarro *et al.*, 2008). Moreover, with the purpose to develop biotechnological sources of trace elements for diet supplementation, microorganisms (e. g. yeast, lactobacilli, and *Spirulina* strains) were proposed as organic matrixes for incorporation of minerals (Mazo *et al.*, 2007; Qin *et al.*, 2007; Slavik *et al.*, 2008). In fact, the addition of inorganic salts into cultivation media enables the biosorption of the mineral ions by the microbial biomass. As a consequence, the biomass becomes enriched with organic forms of trace elements, which are present as complexes with amino acids, proteins, lipids, and polysaccharides (Mazo *et al.*, 2007).

1.1. Minerals requirement in human

1.1.1. Zinc

Minerals, often referred to as micronutrients, are one of the five essentials groups of nutrients needed to sustain life. Micronutrient malnutrition affects more than 50% of the worldwide population (Diplock,

1990; Ramakrishnan, 2002). In particular, Zinc (Zn) deficiency is considered an emerging public health problem in India and in other developing countries. Zn is an important element for growth, present in all eukaryotic organisms, where it is involved in numerous aspects of cellular metabolism (Fukada *et al.*, 2011). It is required for the catalytic activity of approximately 100 enzymes and plays a role in immune function, protein and DNA synthesis, and cell division (Prasad, 2009). A normal daily intake of 15 mg of Zn is required to maintain a steady state because the body has no specialized zinc storage system. Zn absorption is influenced by dietary intake rather than by the Zn status of the organism. As dietary Zn increases, the total amount of absorbed Zinc increases, whereas the the percentage of absorbed mineral declines. The gastrointestinal tract maintains the whole-body Zn homeostasis by adjusting endogeneous Zn losses to the amount adsorbed.

At intakes ≤ 9 mg/die, Zn adsorption occurs primarily by a saturable carrier-mediated process invoving ZIP4, ZnTl, and other transporters. There is no evidence that past Zn intake, or status, influences Zn absorption. Instead, current Zn intake is the chief determinant of Zn absorption (Fukada *et al.*, 2011; King, 2010). ZIP4 and other ZIP family genes expressed in the gastrointestinal tract are upregulated during Zinc restriction. This requires powerful homeostatic control. The transporter ZIP4 is upregulated along the entire gastrointestinal tract by proinflammatory conditions. Intracellular transporters such as ZnT7 influence the transcellular movement of Zn across the enterocyte (Cousins, 2010).

From the enterocyte, where Zn is absorbed by a carrier-mediated process, the mineral is transferred to the portal circulation. Malabsorption of Zn is associated with intestinal disorders, such as Crohn's disease and ulcerative colitis (Scrimgeour *et al.* 2009). The current recommended dietary allowance (RDA) for Zn in the general adult population is 10 mg, whereas during pregnancy and brestfeeding the RDA is raised to 11 mg and 12 mg respectively.

1.1.2. Selenium

Selenium (Se) is another trace mineral essential for humans and animals. Dietary Se primarily exists as selenomethionine and selenocysteine (Tapiero *et al.,* 2003). Plants convert Se mainly into selenomethionine (SeMet), which is incorporated into proteins in place of methionine. Selenocysteine (SeCys) is less abundant and is not significantly incorporated into plant proteins, irrespective of soil Se content. The intermediates of seleno-aminoacids metabolism, such as Se-methyl-selenocystein and gamma-glutamyl Se-methyl-selenocysteine, also occur at low levels. In addition, in some vegetables Se may occur in its inorganic form (selenite). Higher animals are not able to synthetize Se-Met and only Se-Cys was detected in rats supplemented with Se as selenite (Tapiero *et al.,* 2003).

Efficient Se absorption, usually in the range of 50-100%, occurs when the element is supplied as SeMet, while absorption of inorganic Se takes place with lower efficiency. After absorption, Se from SeMet or from inorganic sources is transformed into selenide. Selenide acts the Se donor for the formation of SeCys, the main form of Se in mammal proteins. SeCys is cotranslationally incorporated into polypeptides through a specific mechanism that decodes the UGA codon in mRNA, which normally functions as a stop codon (Lu and Holmgren, 2009). Among SeCys-containing selenoproteins, there are important antioxidant enzymes which help to prevent cellular damage from free radicals, regulate thyroid function, and play a role in the immune system (Rayman, 2005).

Renal regulation is the mode by which whole-body Se is controlled. Se is concentrated in hair and nails and occurs most exclusively in organic compounds. Se has a potent antioxidant activity mediated through its ability to increase the activity of the glutathione peroxidise enzymes (Fritz *et al.*, 2011). Se deficiency is associated with poor health, decreased fertility, cardiovascular diseases, and lowered resistance against viral and bacterial infections. It is also correlated to oxidative stress, since the metal is present in the selenocysteine belonging to the active site of glutathione peroxidase, an

enzyme involved in the protection of cells against hydroperoxides. Se deficiency results in several symptoms associated with oxidative stress, increasing cancer risk (Zhuo *et al.*, 2004) and may contribute to development of a form of heart disease, hypothyroidism, and a weakened immune system (Kurokawa *et al.,* 2013). Severe gastrointestinal disorders may decrease the absorption of selenium, resulting in selenium depletion or deficiency (Stazi *et al.*, 2010). Gastrointestinal problems which impair selenium absorption, such as Crohn's disease or surgical removal of part of the stomach, usually affect absorption of other nutrients as well, and require routine monitoring of nutritional status so that appropriate medical and nutritional treatment can be provided.

The daily allowance of Se indicated for the Italian population by the "Livelli di Assunzione giornaliera Raccomandati di Nutrienti (LARN)", in agreement with the American RDA, is about 55 μg/day for adults, 60 μg and 70μg for pregnancy and brestfeefing, respectively (Food and Nutrition Board-USA, 2000).

1.2. Selenium and Zinc commercial forms

Considering the overall present knowledge, there is good evidence that Se and Zinc deficiencies result in a different kind of pathologies. Furthermore, Zn and Se may also remodel the unavoidable changes related to ageing, thus leading to a possible escape from diseases and subsequent healty ageing as they are involved in the improvement of immune functions, metabolic homeostasis and antioxidant defense (Moccheggiani *et al.*, 2008).

Different inorganic and organic forms of zinc and selenium are currently used in several food supplements, in order to increase the daily intake of these minerals. However, inorganic forms of such minerals, although less expensive, are poorly adsorbed through the gut mucosa and have, therefore, a relatively low systemic effect (Siepmann *et al.*, 2005). Furthermore, a variety of Se and Zn-enriched biological products, including yeast, wheat, fruits, and

vegetables, have been recently developed. The possibility of using some species of probiotics enriched with the two minerals may represent an interesting alternative to these preparations.

2. Materials and Methods

2.1. Bacterial growth and mineral internalization

The strains *Lactobacillus buchneri* Lb26 (DSM 16341) and *Bifidobacterium lactis* Bb1 (DSM 17850) were obtained from Probiotical SpA collection. The strains were kept at -80°C for long term storage, after addition of 20% glycerol. *Lactobacillus* MRS broth was used for the cultures of the *Lactobacillus buchneri* Lb26 strain. Tryptone Phytone Yeast broth (TPY), supplemented with 0.5 g/l L-cysteine-hydrochloride (Scardovi, 1986) was used for the cultures of *Bifidobacterium lactis* Bb1. The strains were sub-cultured thrice at 37°C overnight, before starting the internalization of the minerals.

MRS broth medium had the following composition (g/l):

- Casein peptone, tryptic digest 10.0
- Meat extract 10.0
- Yeast extract 5.0
- Glucose 20.0
- Tween 80 1.0
- K2HPO4 2.0
- Sodium acetate 5.0
- Tri-ammonium citrate 2.0
- MgSO4•7H2O 0.2
- MnSO4•H2O 0.05

Each ingredient was dissolved in distilled water. pH was measured and corrected to 6.65 ± 0.05, if necessary. The liquid medium was dispensed into glass bottles and autoclaved at 121°C for 15 minutes. After sterilization, pH at room temperature (25°C ± 1°C) was 6.60 ± 0.20.

TPY broth medium had the following composition (g/l):

- Tryptone (pancreatic digest of casein) 10.0
- Phytone (papain digest of soybean) 5.0
- Yeast extract 5.0
- Dextrose 10.0
- Tween 80 1.0
- K_2HPO_4 2.0
- $MgCl_2 \cdot 6H_2O$ 0.5
- $ZnSO_4 \cdot 7H_2O$ 0.25
- $CaCl_2 \cdot 2H_2O$ 0.15

Each component was dissolved in distilled water. pH was measured and adjusted to 6.65 ± 0.05. The liquid medium was dispensed into glass bottles and autoclaved at 121°C for 15 minutes. After sterilization, pH at room temperature (25°C ± 1°C) was 6.60 ± 0.10. At the moment of use, a filter-sterilized (0.22 μm) aqueous solution of L-Cysteine hydrochloride solution was added to basal medium to obtain a final concentration of 0.5 g/l.

In order to select the best operative conditions for minerals internalization, several fermentation trials were performed to investigate the effects of different parameters such as inoculation proportion, Se^{4+} concentration (sodium selenite, Na_2SeO_3), Zn^{2+} concentration (zinc sulfate, $ZnSO_4 \cdot 7H_2O$), fermentation time and temperature. Medium optimization started from TPY and MRS broth.

As regard Selenium, a specific protocol that involved the discontinuos addition every 30 minutes of concentrated solutions of the mineral to the cultural broth was followed, in order to preserve cell viability during the

fermentation and the subsequent biomass freeze-drying step. Each fermentation was performed in triplicate. The amount and the rate of internalization of Se and Zn in the bacterial cells was investigated using Atomic Absorption Spectrophotometry (AAS).

2.2. Reagents

Sodium selenite, seleno-L-methionine, seleno-L-Cysteine, zinc sulfate, and zinc gluconate were purchased from Sigma Chemicals (St Louis, MO). All the culture cell reagents were obtained from Invitrogen (Carlsberg, CA). Culture flasks and Petri dishes were supplied by Corning Costar (Cambridge, MA).

2.3. Cell cultures

The bioavailability of Se and Zn internalized by bacterial cells was investigated using human colon adenocarcinoma (Caco-2) cell line, purchased from Leibniz Institute DSMZ (German Collection of Microorganisms and cell cultures; Braunschweig, Germany). Stock cultures were grown in 75 cm^2 culture flasks in Dulbecco modified minimal essential medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) nonessentials aminoacids solution, 15 mM Hepes, and 1% (v/v) penicillin / streptomycin solution (Sambuy *et al.*, 2005). Caco-2 cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, in complete medium (DMEM). The medium was changed every 2 days.

Cells were harvested at 70% confluence and seeded into bicameral chambers (Transwell, 24 mm diameter, 3-μm pore-size, Costar) at a density of 10,000 cells/cm^2. A total of 2.0 ml of medium was added to the well in the basolateral compartment (BL), while 0.5 ml were added in the apical compartment (AL) (Figure 6).

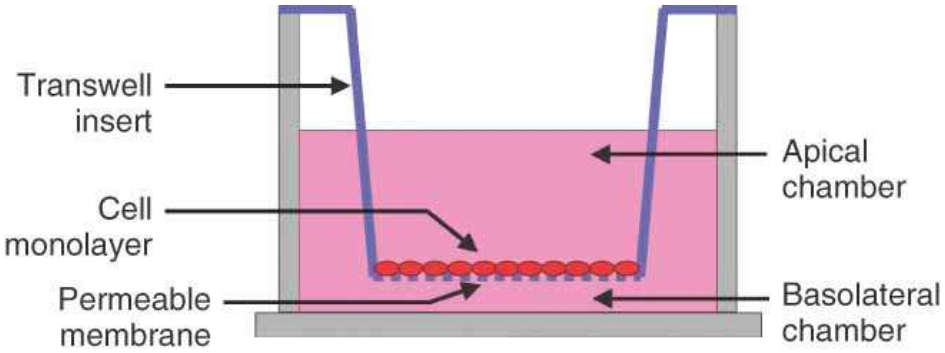


Figure 6. Schematic representation of a Transwell system

Caco-2 viability was assessed with by trypan blue exclusion and was typically >90%. The medium was changed every two days, and cells were used for minerals uptake experiments after 21 days of culture (Figure 7).

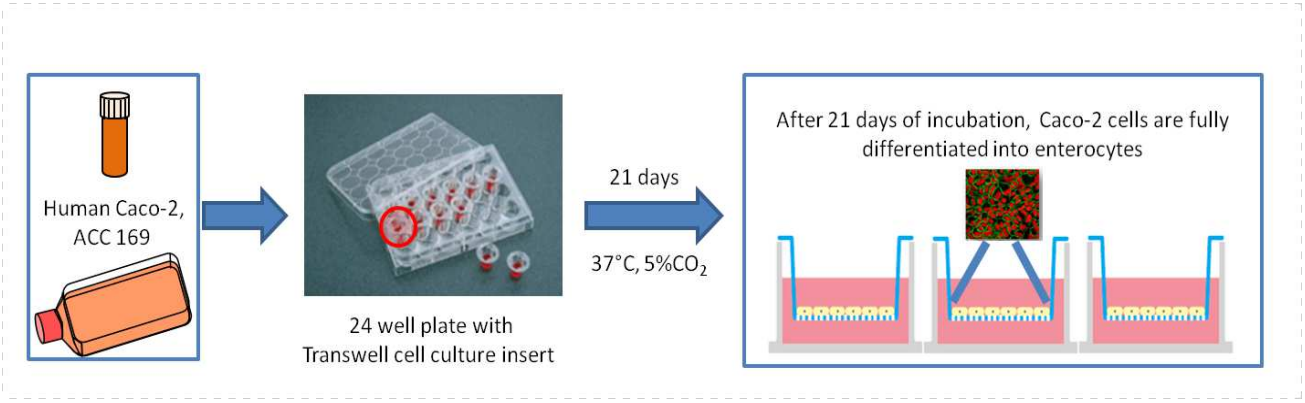


Figure 7. Overview of the experimental scheme used for cells differentiation

The development of functional tight junctions during the differentiation of Caco-2 cells was monitored by determining the transepithelial electrical resistance (TEER) of filter grown cell monolayers after various days of seeding (Jahn *et al.*, 2011). TEER was quantified using the Millicell-ERS apparatus (Millipore) (Foglieni *et al.* 2011).

2.4. Minerals uptake and transportation

Cell monolayers were treated either with solutions containing different selenium and zinc salt, at the same concentration of 1mM (sodium selenite, seleno-L-methionine, seleno-L-cysteine, zinc sulfate, zinc gluconate; Figure 8), or with the individual probiotic bacteria *Lactobacillus buchneri* Lb26 (DSM 16341) and *Bifidobacterium lactis* Bb1 (DSM 17850).

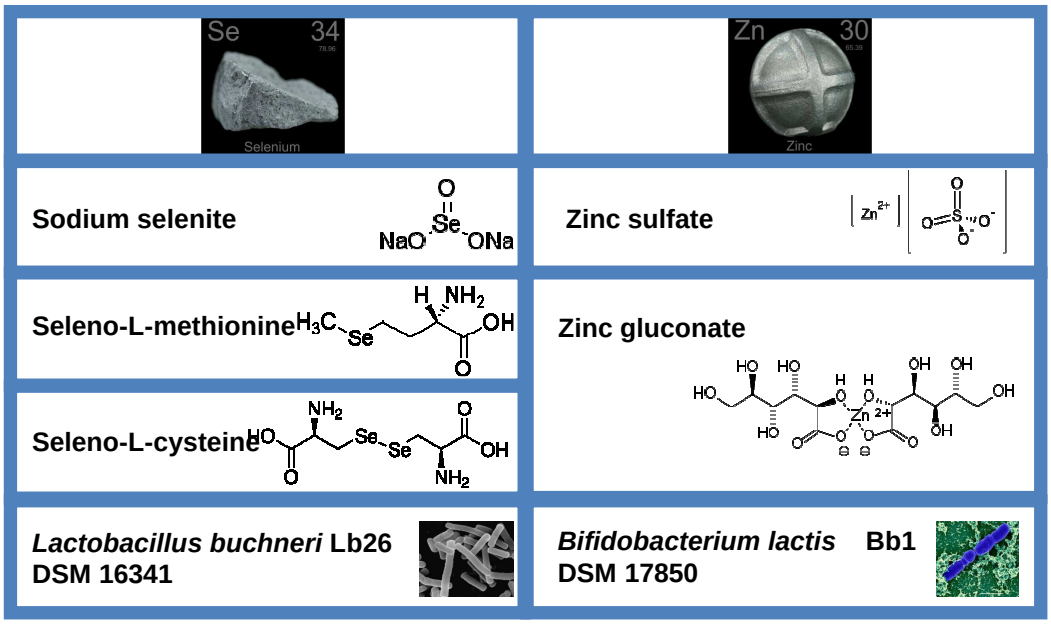


Figure 8. Chemical compounds and strains used in the study

The amounts of Se and Zn internalized by each probiotic were quantified and the strains were resuspended to obtain final solutions containing 1 mM Se or Zn.

Uptake solutions were then added to the AP apical chamber of the Transwell, and cell cultures were incubated at at 37°C for 24 hours in a humidified atmosphere. At the end of incubation, medium samples from the AP and BL compartments were collected for mineral analysis, then the filter insert was removed, and the cell surface was washed twice with phosphate buffer saline to remove residual medium. The microporous membrane with the cell monolayer was cut, the cells were treated with trypsin, then harvested.

Cell viability after 24 hours of exposure to the uptake solutions was assessed by trypan blue exclusion. The experiment was carried out in triplicate.

2.5. Analysis of minerals

Se ans Zn were quantified in bacterial biomass of *Lactobacillus buchneri* Lb26 and *Bifidobacterium lactis* Bb1, in medium samples from the AP and BL Transwell compartments, and in Caco-2 lysed cells. All the samples were prepared for mineral analysis by mineralization with concentrated nitric acid and hydrogen peroxide. Se concentration was determinated by hydride generation atomic absorption spectrometry using a Shimadzu AA-6200 spectrophotometer coupled with a Younglin HG 8000 hydride generator (AAS) (Figure 9). Zn concentration was determinated by flame atomic absorption spectrometry using a Shimadzu AA-6200 spectrophotometer. All reagents and standard solutions were purchased from Sigma Chemical.

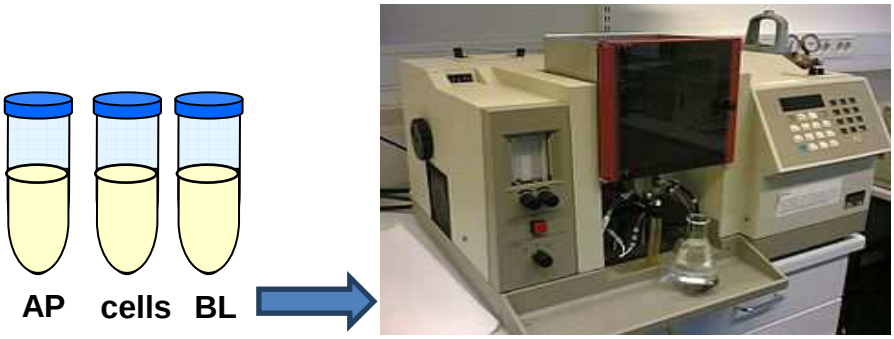


Figure 9. Samples from AP and BL compartments and cells to be analyzed by AAS

3. Results

3.1. Bacterial growth and mineral internalization

In order to develop mineral-enriched probiotics, appropriate operative conditions to enrich of Selenium and Zinc the biomass of *Lactobacillus buchneri* Lb26 (DSM 16341) and *Bifidobacterium lactis* Bb1 (DSM 17850), previously selected for their ability to internalize selenium and zinc, respectively, were assessed. In order to select the best operative conditions for minerals internalization, several fermentation trials were performed to investigate the effects of different parameters, such as medium composition, inoculation proportions, concentration of Se^{4+} or Zn^{2+} (given as sodium selenite Na_2SeO_3 or zinc sulfate $ZnSO_4 \cdot 7H_2O$, respectively), fermentation time, and temperature (Tables 2 and 3).

	Fermentation trial						
	I	II	III	IV	V	VI	VII
% inoculum, %	2.5	2.5	2.5	5	5	5	5
Temperature, °C	37	37	35	37	37	37	37
Incubation time, h	24	24	24	24	24	24	24
Selenite addition at t_0, mg/l	0	20	20	20	10	/	/
Selenite addition in steps, mg/l	/	/	/	/	/	20	40
LB26 viable cell count, UFC/g	$198x10^9$	$3x10^9$	$1.5x10^9$	$5x10^9$	$25x10^9$	$48x10^9$	$35x10^9$
Se in freeze dried cells, mg/g	0.0002	0.015	0.011	0.034	0.029	0.86	1.89

Table 2. Improvement of the fermentative process for Se uptake by *L. buchneri* Lb26

	Fermentation trial							
	I	II	III	IV	V	VI	VII	VIII
Inoculum, %	2.5	2.5	2.5	2.5	5	2.5	2.5	2.5
Temperature °C	37	37	37	37	37	35	37	37
Incubation time, h	15	15	15	15	15	15	15	24
Zinc addition at t0, mM	0	5	10	15	5	5	/	/
Zinc addition in steps, mM	/	/	/	/	/	/	5 mM at 4 h	5 mM at 15 h
Bb1 viable cell count, UFC/g	$420x10^9$	$55x10^9$	$24x10^9$	$2\ x10^8$	$51x10^9$	$42x10^9$	$78x10^9$	$230x10^9$
Zn in freeze dried cells, mg/g	0,002	19.8	21.2	32.1	17.8	18.5	13.2	3.9

Table 3. Improvement of the fermentative process for Zn uptake by *B.lactis* Bb1.

In the best enrichment procedure, *L. buchneri* Lb26 and *B. lactis* Bb1 were sub-cultured three times at 37°C for 16 h in MRS and TPY medium, respectively, before starting the internalization process of the minerals. After fermentations trials aiming to optimize the medium and the process for selenium uptake, the following Selenium Enrichment medium for of *L. buchneri* Lb26 was developed (g/l):

- Casein peptone, tryptic digest 10.0
- Soy peptone 8.0
- Yeast extract 10.0
- Sucrose 60.0
- Tween 80 1.0
- K2HPO4 2.0
- Sodium acetate 5.0
- Tri-ammonium citrate 2.0
- $MgSO_4 \cdot 7H_2O$ 0.2
- $MnSO_4 \cdot H_2O$ 0.05

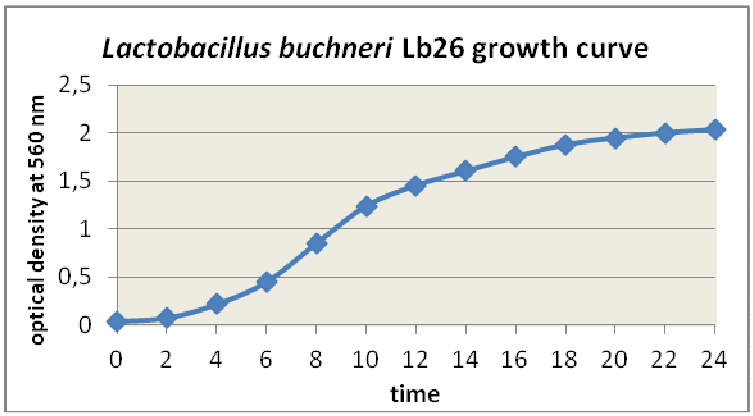
The fermentation was performed at the temperature of 37°C for 24 hours, in aerobic conditions, without pH controlling and with a stirring speed setted at 50 rpm. Since the viability of the culture resulted affected by Selenium (Table 2), to preserve cell viability for the entire duration of the fermentation and subsequent freeze-drying of bacteria, a specific protocol that involved the discontinuos addition every 30 min of a filtered concentrated solution of sodium selenite to the cultural broth was exploited, to provide a total 40 mg/l selenite.

With regard to the strain *Bifidobacterium lactis* Bb1 (DSM 17850), the following cultural broth for Zinc enrichement was developed (g/l):

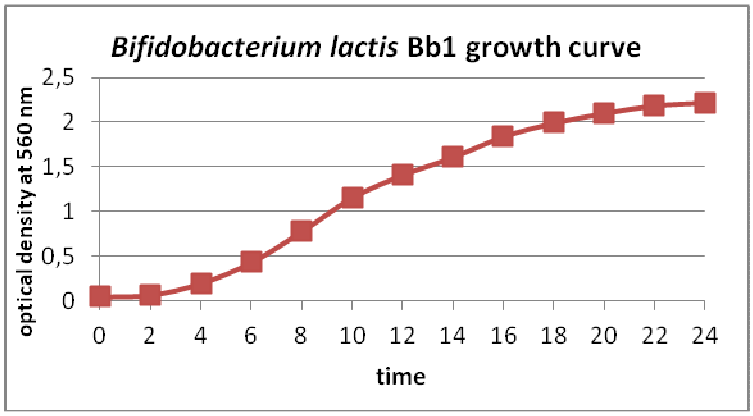
- Casein peptone, tryptic digest 20.0
- Soy peptone 8.0
- Yeast extract 10.0
- Destrose 40.0
- Tween 80 1.0
- K2HPO4 2.5
- $CaCl_2 \cdot 2H_2O$ 0.15
- $MgCl_2 \cdot 6H_2O$ 0.5
- $ZnSO_4 \cdot 7H_2O$ 0.9

The fermentation was performed at 37°C for 15 hours, in anaerobic conditions, with a pH range neutralization setted at 5.90 ± 0.1 and with a stirring speed setted at 50 rpm. In the case of *Bifidobacterium lactis* Bb1, it was possible to add at the begin of the fermentation all the Zinc to be internalized by the cells, due to the low toxicity of the mineral versus this strain (Table 3).

The growth curves of the strains under the above described culture conditions are reported in Figure 10 A and B



A



B

Figures 10. Growth curves of the strains *L.buchneri* Lb26 and *B.lactis* Bb1 during the fermentation processes aimed to internalize Se and Zn, respectively.

For both strains, at the end of the fermentation, cells were harvested, washed with sterile water, and freeze dried, after addiction of a solution of sucrose. After freeze-drying, viable cell count was evaluated and the rate of internalization of Se and Zn in the bacterial cells was investigated using Atomic Absorption Spectrophotometry (AAS) (Table 4).

Strain	Viable cell counts	Minerals
L. buchneri Lb26	35 x 10^9 CFU/g	1.9 mg Se/g
B. lactis Bb1	55 x 10^9 CFU/g	19.8 mg Zn^{2+}/g

Table 4. Viable cell counts and dosage of Se and Zn in lyophilized formulations of Se enriched *Lactobacillus buchneri* Lb26 and Zn-enriched *Bifidobacterium lactis* Bb1. Values are means, n = 3, SD always < 10%.

The highest value of selenium internalized by *L.buchneri* Lb26 was found using the parameters of the test VII (addition of Se in steps, total 40mM) described in Table 2. This protocol allowed to get a good viable cell count in the freeze-dried product.

As regard zinc, the product obtained from test II represented a good compromise between Zn content and *B. lactis* Bb1viable cell count (addition of Zn 5mM at t0) described in Table 3, and was utilized for the subsequent uptake tests.

3.2. Se and Zn uptake through Caco-2 cells

To evaluate the effectiveness of Se- and Zn-enriched probiotics as mineral carriers, Se or Zn uptake through Caco-2 cells was assayed. Caco-2 cell monolayers were grown in Transwell wells, and were put in contact with different uptake solutions, added to the AP apical chamber. Uptake solutions contained the same concentration (1 mM) of selenium and zinc (sodium selenite, seleno-L-methionine, seleno-L-cysteine, zinc sulfate, or zinc gluconate) or appropriate amounts of Se-enriched *L. buchneri* Lb26 or Zn-enriched *B. lactis* Bb1, similarly providing 1 mM Se or Zn. After 24 h of incubation at 37°C, the minerals were quantified within Caco-2 cells and in AP and BL compartments, to determine the extent of internalization and translocation through the monolayer.

Integrity of Caco-2 monolayer and development of functional tight junctions during the differentiation of Caco-2 cells are essential prerequisites for this approach. In fact, the amount of zinc and selenium found in the Transwell BL basolateral compartment can be exclusively attributed to the mineral uptake by Caco-2 cells from the AP apical compartment, and the subsequent diffusion within the cell and the transportation across the cell basolateral membrane. Thus, transepithelial electrical resistance (TEER) of filter grown cell monolayers was measured before the uptake experiment. All

the Caco-2 monolayers grown in transwell wells exhibited adequate TEER values, always higher than 800Ω/cm^2 (Table 5).

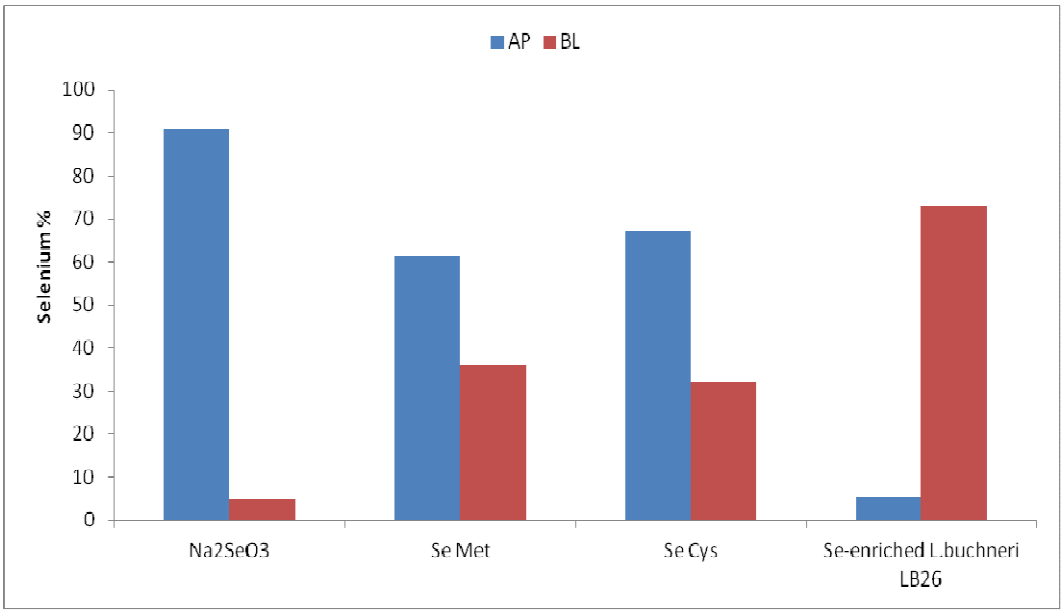
At the end of the uptake experiment, the microporous membrane bearing the cell monolayer was removed, washed, and treated with trypsin to harvest Caco-2 cells and assay their viability through trypan blue exclusion. Caco-2 cell viability at the end of the experiments was never <87%.

	1	2	3	4	5	6
A	1480	1320	1498	1388	1249	1445
B	1409	1378	1478	1298	1255	1450
C	1470	1355	1312	1303	1268	1380
D	1367	1462	1440	1418	1207	1341

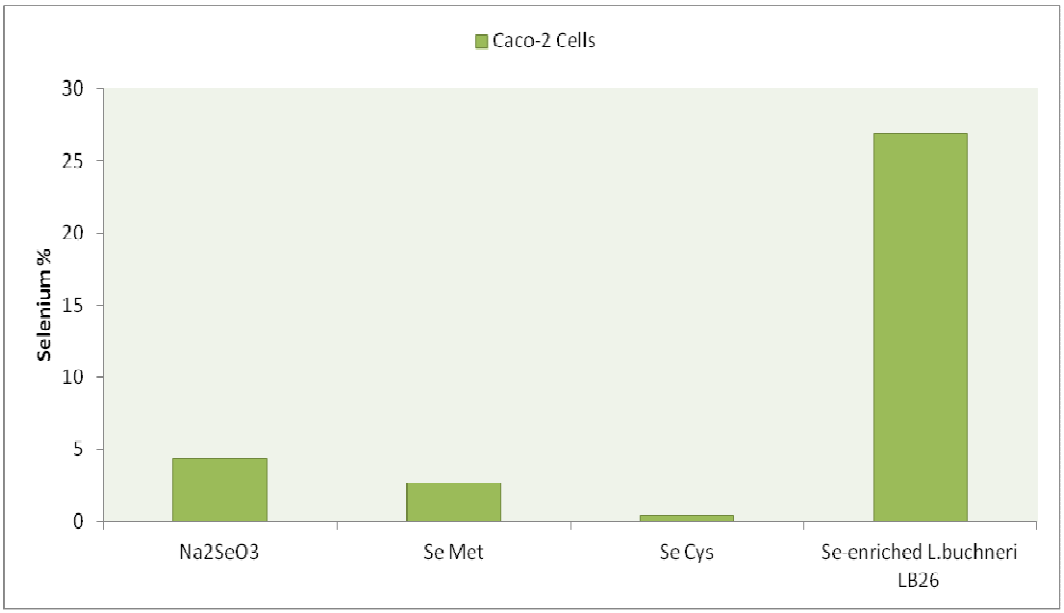
Table 5. TEER values in watt/well measured after 24h of stimulation in 24 well plate

3.2.1 Selenium uptake

Results of selenium uptake are reported in Figure 11 A and B, where the amount of selenium residually present in the AP chamber, translocated to the BL chamber, and internalized within Caco-2 cells is reported as the percentage in relation to the amount initially added to the AP chamber of the Transwell.



A



B

Figures 11. Distribution of Se in AP and BL compartments and uptake by Caco-2 cells. Values are means, n = 3, SD always < 10%

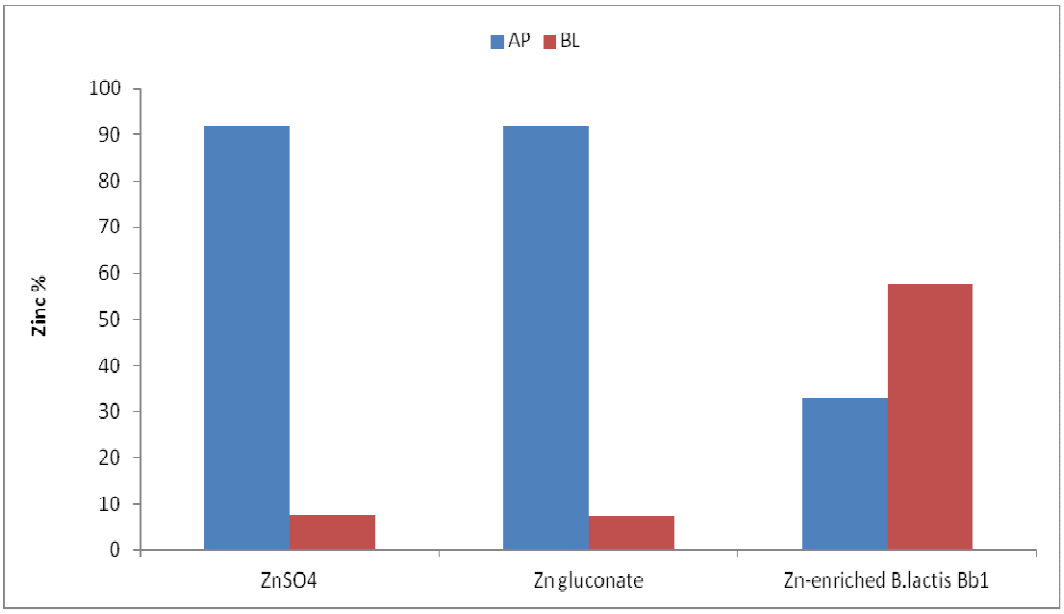
Mineral found in the AP chamber corresponds to the portion not absorbed by Caco-2 cells, whereas mineral in the BL compartment has been transported through Caco-2 cells. The results of Se uptake suggest that the organic form accumulated by *L. buchneri* Lb26 is the most available one, being transported to the BL compartment at the highest percentage.

In particular, 69.3% of probiotic associated Se was found in the BL chamber, while only 32.2% of Se-L-Cysteine and 35.9% of Se-L-methionine was able to cross Caco-2 cells. Sodium selenite was present at only 4.9% in the BL compartment and 4.3% inside of Caco-2 cells, meaning an overall AP absorption by the cells of <10%. Another interesting point is the fact that only 5.1% of probiotic Se was not absorbed by Caco-2 cells from the Transwell AP chamber, compared with 67.5% , 61.4%, and 90.8% of Se-L-Cysteine, Se-L-methionine, and sodium selenite respectively, found in the AP compartment at the end of the incubation.

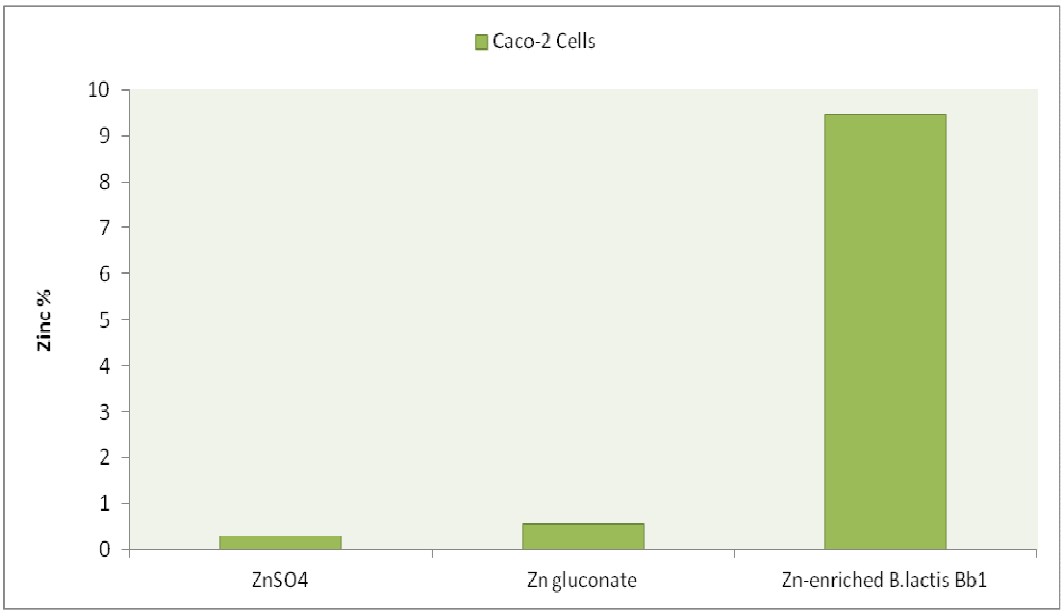
Selenium internalization in Caco-2 cells was 65.0, 9.4, and 5.9 fold higher for the Se-enriched probiotic, compared with Se-L-Cysteine, Se-L-methionine and sodium selenite.

3.2.2. Zinc uptake

Results of zinc uptake are reported in Figure 12 A and B, where the amount of Zinc residually present in the AP chamber, translocated to the BL chamber, and internalized within Caco-2 cells is reported as the percentage in relation to the amount initially added to the AP chamber of the Transwell. Mineral found in the AP chamber corresponds to the portion not absorbed by Caco-2 cells, whereas mineral in the BL compartment has been transported through all Caco-2 cells.



A



B

Figures 12. Distribution of Zn in AP and BL compartments and uptake by Caco-2 cells. Values are means, n = 3, SD always < 10%

As shown in Figure 12, organic form accumulated by *B.lactis* Bb1 is the most available, being transported to the BL compartment at the highest percentage.

In particular, 57.6% of probiotic Zn was found in the BL chamber, while only 7.4% of Zn gluconate and 7,7% of Zn sulfate was able to cross Caco-2 cells. Consistently, 32.9% of probiotic Zn was not absorbed by Caco-2 and

therefore remained in the AP chamber, compared with 92.0% of Zn gluconate and Zn sulfate. With regard to absorption by Caco-2 cells, probiotic Zn was absorbed 17.0 and 31.5 times more efficiently than Zn gluconate and Zn sulfate, respectively.

4. Discussion

Micronutrient malnutrition affects more than 50% of the worldwide population (Diplock, 1990; Ramakrishnan, 2002). Different inorganic and organic forms of Zn and Se are currently used in numerous food supplements with the purpose of increasing the daily intake of these minerals. Inorganic salts have a very low bioavailability, whereas organic forms are generally characterized by a higher systemic value (Siepmann *et al.*, 2005; Qin *et al.*, 2007). The innovative opportunity of using certain species of probiotics enriched with one of the two minerals may represent an interesting alternative to these preparations. Diet supplementation with beneficial bacteria able to internalize Zn and Se could represent a new application of probiotics.

In this work, the ability of two probiotic strains to internalize Zn and Se was described and the bioavailability of the two minerals was studied. A Caco-2 cell line was purposely employed to resemble human colonic cells as much as possible, as they are fully equipped with enzymatic and transportation systems commonly used by Zn and Se (Sambuy *et al.*, 2005). In particular, the transportation of different forms of Se and Zn (probiotic, organic, and inorganic) across a Caco-2 monolayer model was assessed to evaluate their absorption in a Transwell system.

The results showed that these different compounds are transported at considerably different extents across the Caco-2 cell monolayer, thereby suggesting a higher *in vivo* bioavailability of the probiotic forms compared to the inorganic and even the organic molecules used. Even if fairly unexpected, the overall bioavailability of the organic Zn tested was very similar to the inorganic salt used, namely zinc sulfate. At any rate, the Se and Zn forms

internalized by *Lactobacillus buchneri* Lb26 (DSM 16341) and *Bifidobacterium lactis* Bb1 (DSM 17850), respectively, showed the highest absorption and cell diffusion across Caco-2 cells, therefore suggesting a notably improved *in vivo* bioavailability and systemic relevance.

Further *in vivo* assessments are necessary to confirm the significantly higher bioavailability of probiotic Zn and Se, compared with organic forms with a widespread commercial use. These results suggest the opening of new horizons in micronutrient supplementation, thus providing more bioavailable forms of both Se and Zn and embodying new nutraceutical applications of probiotic bacteria.

OXALATE DEGRADING PROBIOTIC BACTERIA

1. Background

1.1. Oxalate homeostasis in the gut

Oxalic acid is a highly oxidized, strong organic acid that is widely distributed in nature, occurring both in plants and animals. It generally accumulates as a metabolic end product, in the form of free acid. It can act as a chelator of cations and it is often found as soluble sodium or potassium oxalate, or in precipitated form as insoluble calcium oxalate (Lung *et al.,* 1994), which can accumulate as microscopic crystals in the plant tissues, sometimes contributing as much as 80% of the dry weight of the plant (Webb, 1999).

Oxalic acid and its oxalate salts also occur in the blood (plasma) and urine of animals and humans. The oxalate found in humans partially comes from diet, ingesting the oxalate-containing plant material in the form of foods such as strawberries, spinach, rhubarb, beets, nuts, wheat, bran, chocolate, tea, and coffee (Duncan *et al.,* 2002). A further amount of oxalate is formed endogenously, in the liver, through the metabolism of glycine, glyoxylate, and ascorbic acid (Holmes and Assimos, 1998). However, the extent of the exogenous and endogenous contribution to the oxalate concentration in the gut are still under debate (Ferraz *et al.,* 2009).

Humans lack the enzymes that are needed to metabolize oxalate, and this potentially toxic compound is, therefore, remuved chiefly in three ways:

- it can be adsorbed into the urinary tract and excreted in urine;
- it can combine with calcium, forming insoluble calcium oxalate, which is eliminated in faeces;
- it can be be degraded by microorganisms present in the gastrointestinal tract (GIT).

The relative amounts of calcium and oxalate are important factors affecting the rate of oxalate adsorption and urinary excretion (Camperi *et al.*, 2001).

It has been shown that, in healthy individuals, an increased intake of dietary oxalate can significantly enhance the urinary excretion (Massey *et al.,* 1993). Hatch *et al.,* (1993) measured the transport of ^{14}C-oxalate *in vitro* across the various regions of rat and rabbit intestine. They found that in the ileum, jejunum, and proximal colon there was the net movement of oxalate from the serum to the gut mucosa, suggesting that oxalate would be secreted into these regions of the gut. Coversely, in the distal colon a trend of oxalate adsorption was observed (movement from the mucosa to serum). An implication of this finding would be that levels of oxalate found in the GIT might vary along these regions, influencing the local nutrient availability, and the settled oxalate-degrading microorganisms.

1.2. Hyperoxaluria and kidney stone disease

High levels of oxalate in humans can have a detrimental effect. It can cause a range of medical pathologies including hyperoxaluria and renal failure (Hoppe *et al.,* 2009), calcium oxalate urolithiasis (Camperi *et al.,* 2001), and cardiomyopathy (Van Driessche *et al.*, 2007).

Hyperoxaluria is characterized by extremely high levels of urinary oxalate, which can lead to urolithiasis (stone formation), that occur mostly in kidneys, but also anywhere in the urinary tract including the uretra and bladder (Duncan *et al.,* 2002). The process of kidney stone formation involves the formation of crystals that separate from urine and accumulate on the inner surfaces of the urinary tract. Kidney stone disease is a worldwide problem affecting between 3% and 20% of people, and many patients present relapse.

The development of calcium oxalate stones has been shown to be a multifactorial process, which is influenced by environmental and genetic factors (Knight *et al.,* 2007). Risk factors are race, diet, climate,season, and

gender, having men a higher risk of developing kidney stone disease (10%) than women (5%).

1.3. Oxalate degrading gut bacteria

GIT bacteria degrade many dietary substances that cannot be digested by humans, oxalate being one of these molecules. Oxalic acid is a dicarboxylic acid, toxic at high levels and it is not generally the primary source of energy for most bacteria, as it has a low energy yield when catabolized. However, two major groups of oxalate-degrading GIT bacteria can be identified (Sahin, 2003): the "generalist oxalotrophs", which do not depend entirely on oxalate as an energy source and can ferment also other substrates, and the "specialist oxalotrophs",which use oxalate as their sole or major carbon and energy source.

Oxalate degradation by bacteria occurs via both aerobic and anaerobic respiratory pathways (Sahin, 2003). During aerobic growth, oxalate is metabolized to produce CO_2 and formate, than formate dehydrogenase oxides the latter compound. Anaerobic bacteria, such as those found in the GIT, however, are not able to oxidize the formate and thus this accumulates as major end product of the catabolism of oxalate (Cornick and Allison, 1996).

Oxalobacter formigenes was the first oxalate-degrading obligate anaerobe to be described in humans (Allisons *et al.,* 1985) and is the model organism in which anaerobic oxalate degradation has been studied. It has three enzymes involved in the catabolism of oxalic acid. The process begins with the uptake of the extracellular oxalate by the membrane associated formate-oxalate antiporter OxlT, encoded by the *oxlT* gene (Abe *et al.,* 1996;Anantharam *et al.,* 1989; Ruan *et al.,* 1992). The *frc* gene encodes formyl-CoA transferase Frc, which activates the intarcellular oxalatefrom oxalyl-CoA (Baetz and Allison, 1990). This is decarboxylated in a thiamine PP_i-dependent reaction by the oxalyl-CoA decarboxylase Oxc, enzyme expressed by *oxc* gene. Formate and carbon dioxide are the end products, and the formate-oxalate antiporter, OxlT, catalyzes the export of the intracellular formate out of the cells. The

decarboxylation of oxalate generates a proton pump gradient, which produces one ATP molecule when it is coupled with oxalate-formate transport (Ruan *et al*., 1992). *O. formigenes* uses oxalate as an exclusive source of energy and thus may be defined as a "specialist oxalotrophs".

Other oxalate-degrading bacteria isolated from human feces are:

- *Enterococcus faecalis,* a gram positive intestinal commensal (Hokama *et al*. 2000);
- *Providencia rettgeri,* a gram negative facultative anaerobe studied by Hokama *et al*. (2005);
- *Eubacterium lentum,* that belongs to the most common bacterial genus found in the human GIT after the *Bacteroides* (Ito *et al.* 1996a,b);
- *Escherichia Coli.*

Often these bacteria isolated from feces initially show an improved growth on an oxalate-enriched medium and soon lose their ability to degrade oxalate after some sub-cultivation. It is very difficult to sustain and observe the capacity to degrade oxalate under the currently used *in vitro* conditions.

1.4. Oxalate degrading *Lactobacillus* and *Bifidobacterium* species

Recently, there has been an increased research interest in exploiting the oxalate-degrading capabilities of *Lactobacillus* and *Bifidobacterium* species in order to develop probiotics to alleviate urolithiasis disease. Table 6 gives a summary of the major studies reporting oxalate degradation by strains of *Lactobacillus* and *Bifidobacterium.* It has been noted that oxalate degradation in the lattobacilli and bifidobacteria is both species- and strain-specific.

In vitro experiments by Camperi *et al*. (2001) showed that the *Lactobacillus* and *Bifidobacterium* strains tested were "generalist oxalotrophs" since they were unable to utilize oxalate as a principal carbon source and could only degrade oxalate when the growth media contained lactose or glucose as well as oxalate. However, a polymerase chain reaction (PCR) investigation of these isolates, using primers designed for the genes *oxlT, frc,* and *oxc* of *O. formigenes*, did not detect the presence of orthologs of these genes in any of the

oxalate-degrading *Lactobacillus* and *Bifidobacterium* species reported. Federici *et al.* (2004), using a different set of primers to the *O. Formigenes oxc* gene, successfully amplified a homologue from an oxalate degrading strain of *Bifidobacterium lactis.* This *oxc* gene encoded a functional oxalyl-CoA decarboxylase enzyme when the recombinant protein was expressed in *E. coli*, and the protein cross-reacted with anti - *O. formigenes* oxalyl-CoA decarboxylase antibodies. However, no other proteins involved in the mechanisms of oxalate degradation in *Bifidobacterium* species have been reported.

Bacteria[a]	**Reference**
Lactobacillus plantarum (*n*=1/1)	Camperi *et al*. (2001)
Lactobacillus brevis (*n*=1/1)	
Lactobacillus acidophilus (*n*=1/1)	
Bifidobacterium infantis (*n*=1/1)	
Bifidobacterium lactis DSM *10140* (*n*=1/1)	Federici *et al*. (2004)
Bifidobacterium animalis ATCC 27536 (*n*=1/1)	
Bifidobacterium breve MB 238 (*n*=1/2)	
Bifidobacterium longum MB282 (*n*=4/5)	
Bifidobacterium infantis MB 57(*n*=1/2)	
Bifidobacterium adolescentis MB238 (*n*=1/1)	
Lactobacillus casei (n=2/31*)*	Kwak *et al.* (2006)
Lactobacillus acidophilus (*n*=1/1)	Azkarate–Peril *et al.* (2006)
Lactobacillus gasseri Gasser AM63^T *(n*=1/1*)*	Lewanika *et al.* (2007)
Lactobacillus acidophilus (*n*=32/32)	Turroni *et al.* (2007)
Lactobacillus gasseri (n=6/6*)*	
Lactobacillus plantarum (*n=3/7*)	
Lactobacillus casei (n=2/3*)*	
Lactobacillus rhamnosus (n=2/2*)*	
Lactobacillus salivarius (n=1/1*)*	
Lactobacillus gasseri ATCC 33323 *(n=7/9)* (previously known as *L. gasseri Gasser AM63T)*	Azkarate–Peril *et al. (2008)*

Table 6. Reported oxalate–degrading *Lactobacillus* and *Bifidobacterium* species
[a] (n=x/y): Bacterial numbers in brackets indicate how many isolates showed the capability to degrade oxalate, (x) relative to the total number of isolates tested in the study (y).

Oxalate catabolism in *Lactobacillus* spp. has been deeply investigated. Genes encoding the oxalate-degrading enzymes Frc and Oxc were identified in *Lactobacillus acidophilus* NCFM *(*Azkarate–Peril *et al.,* 2006*).* It was shown that oxalate-dependent induction of these genes occurred only when the cells were first adapted to subinhibitory concentrations of oxalate under mildly acidic conditions (pH 5.5). The molecular mechanism of oxalate degradation in *Lactobacillus gasseri* Gasser AM63^{T} has also been investigated (Lewanika *et al.,* 2007). The presence of *frc* and *oxc* genes was confirmed: they were shown to be enclosed in a operon induced by the presence of oxalate (Azkarate–Peril *et al.,* 2006). A study by Turroni *et al*. (2007), identified a group of *Lactobacillus* spp. that could degrade oxalate. The presence of *oxc* and *frc* genes was shown in all the analysed *L. acidophilus* and *L. gasseri* strains that degraded more than 50% oxalate. Specific functional characterization of the Frc and Oxc enzymes from one of the *L. acidophilus* isolates confirmed that these enzymes were responsible for oxalate degradation.

In Table 6 are collected data from different studies on oxalate degradation in *Lactobacillus* spp.: all the tested strains of *L. acidoph*ilus were able to degrade oxalate efficiently, conversely only 14 out 16 of *L. gasseri*, 2 out 31 strains of *L. casei*, and 3 out 7 *L. plantarum* consumed the substrate. This inter-species and inter-strains variation deserves deeper investigations in an evolutionary perspective, and must to be taken into account for an appropriate selection of bacterial isolates to be exploit for therapeutic applications.

2. Materials and Methods

2.1. Reagents, strains, media, and growth conditions

All probiotic strains screened for the capability to degrade oxalate, belonging to the genera *Lactobacillus* and *Bifidobacterium*, were obtained from the collection of Probiotical S.p.A. (Table 7). The complete taxonomic classification of strains was performed on the basis of biochemical features, species-specific PCR, and Pulsed Field Gel Electrophoresis (PFGE), a

method able to discriminate different strains within the same species. *Oxalobacter formigenes* was purchased from DSM collection (DSM 4420) has been used as positive control of oxalate-degrading strain.

Bacterial species	Strain	International Collection Deposit number
Lactobacillus paracasei	LPC 09	DSM 24243
Lactobacillus gasseri	LGS 01	DSM 18299
Lactobacillus gasseri	LGS 02	DSM 18300
Lactobacillus acidophilus	LA 07	DSM 24303
Lactobacillus acidophilus	LA 02	DSM 21717
Lactobacillus plantarum	LP 01	LMG-P 21021
Lactobacillus reuteri	LRE 03	DSM 23879
Lactobacillus reuteri	LRE 02	DSM 23878
Bifidobacterium breve	BR 03	DSM 16604
Bifidobacterium animalis	//	DSM 20104
Bifidobacterium longum	BL 02	DSM 24697
Bifidobacterium longum	BL 03	DSM 16603
Lactobacillus rhamnosus	LGG	ATCC 53103
Lactobacillus reuteri	LRE 04	DSM 23880
Lactobacillus rhamnosus	LR 06	DSM 21981
Bifidobacterium lactis	BA 05	DSM 18352
Lactobacillus rhamnosus	LR 04	DSM 16605
L. delbrueckii subsp. *delbrueckii*	LDD 01	DSM 22106

Table 7. Bacterial strains tested in the screening of oxalate degrading activity

All reagents were supplied by Sigma Aldrich (USA) or Difco Laboratories (USA). *Lactobacillus* and *Bifidobacterium* strains were grown in De Man,

Rogosa and Sharpe (MRS) broth (de Man *et al.*, 1960); for *Bifidobacteria* the broth was added with 0.5 g/l of L-Cysteine hydrochloride. Cultures were incubated at 37°C in anaerobic conditions (GasPak System) using Anaerocult A kits. For long-term storage, over-night MRS cultures were supplemented with 25% glycerol as cryoprotectant and maintained at -80°C. Before experimental use, the strains were subcultured thrice for 24 h.

O. formigenes was cultured, according to Hungate (1969) in medium 419 (DSMZ, Germany) at 37°C for 48 hours, with the only exception that Na-oxalate was substituted with NH_4-oxalate (Sigma-Alidrich, USA).

The 419 medium was prepared according to the following recipe (g/l):

▪	K_2HPO_4		0.250 g
▪	KH_2PO_4		0.250 g
▪	$(NH4)_2SO4$		0.500 g
▪	$MgSO_4$ x 7 H2O		0.025 g
▪	Na-acetate		0.820 g
▪	Yeast extract		1.000 g
▪	Trypticase		1.000 g
▪	Na-oxalate		5.000 g
▪	Resazurin		1.000 mg
▪	Na_2CO_3		4.000 g
▪	Cysteine-HCl x H_2O		0.500 g
▪	Trace element sol. SL-10		1.000 ml
▪	Distilled water	to	1,000 ml

Each ingredient was dissolved in distilled water, except carbonate and cysteine; pH was measured and adjusted to 6.8 ± 0.05, if necessary. Solution was heated to boiling and then cooled to room temperature under 100% CO_2 gas atmosphere. Solid carbonate and cysteine were added and then pH was measured and corrected to 6.8 ± 0.05, if necessary. The liquid medium was

dispensed under 100% CO_2 in anaerobic vessels and autoclaved at 121°C for 15 minutes.

Trace element solution SL-10 was prepared according to the following recipe (g/l):

- HCl (25%; 7.7 M) 10.0 ml
- $FeCl_2$ x 4 H_2O 1.5 g
- $ZnCl_2$ 70 mg
- $MnCl_2$ x 4 H_2O 100 mg
- H_3BO_3 6 mg
- $CoCl_2$ x 6 H_2O 190 mg
- $CuCl_2$ x 2 H_2O 2 mg
- $NiCl_2$ x 6 H_2O 24 mg
- Na_2MoO_4 x 2 H_2O 36 mg
- Distilled water 990.00 ml

The salt $FeCl_2$ was first dissolved in the HCl, then diluted in water; the other salts were then added and dissolved. Finally the solution was made up to 1000 ml.

2.2. Colture conditions for oxalate-degrading tests

The oxalate-degrading tests were performed in triplicate. Fresh bacteria were inoculated in a semidefined medium (SM) as described by Campieri *et al*. (2001). The SM medium was prepared according to the following recipe mixing 5 ml of filtered sterilized ammonium oxalate solution (20 mmol/l ammonium oxalate and 40 g/l dextrose) with 5 ml of base medium.

The base medium was prepared according to the following recipe (g/l):

- Protease peptone 20.0 g

- Yeast extract 10.0 g
- Tween 80 2.0 ml
- KH_2PO_4 4.0 g
- Na-acetate 10.0 g
- Diammonium hydrogen citrate 4.0 g
- $MgSO_4 \cdot 7H_2O$ 0.1 g
- $MnSO_4$ 0.1 g

Each ingredient was dissolved in distilled water. The liquid medium was dispensed into glass tubes and autoclaved at 121°C for 15 minutes.

Lactobacillus and *Bifidobacterium* strains and *O. formigenes* were inoculated into SM medium in order to obtain an initial absorbance at 600 nm of approximately 0.10 OD (Optical Density) and were incubated anaerobically at 37°C for 24 hours. SM medium without the inoculum was used as negative control. At the end of incubation, the samples were centrifuged at 6000 rpm for 10 minutes in order to discharge the bacterial biomass. Supernatants were filtered by Minisart® 0.45 μm filter (Sartorius, Germany) and stored at -18 °C.

2.3. Sample preparation

Before HPLC analysis, supernatants were purified using Strata-X-A 33u Polymeric Strong Anion (500mg/6ml) SPE column (Phenomenex, UK), according to manufacturer instruction. SPE column activation step was performed with 1 ml of methanol and the conditioning step was performed with 2 ml of sodium formate 10 mM. The pH of the supernatant value was adjusted to 6.00 ± 0.05 with 0.1M NH_4OH. Subsequently, 1 ml was loaded into the column. Washing step was performed with 1 ml of sodium acetate followed by 1 ml of methanol. Sample elution was done with 1 ml of 1 M HCl and 1 ml of 3 M HCl. Before injection, the pH was adjusted to 6.0 ± 0.05

using 0.1 M NH_4OH. All chemicals were of HPLC grade and were supplied by Sigma Aldrich (USA).

2.4. HPLC analysis

Chromatographic analysis were performed with a Shimadzu HPLC system consisting of a CTO-20A oven, a SPD-M20A UV visible diode array detector, a DGU-20A5 degaser, and a LC-20AT liquid chromatography pump. Peak areas were calculated using LabSolution LC single/PDA software. The HPLC column used was a Synergi Hydro-RP column, 4 μm 250 mm x 4,6 mm i.d. (Phenomenex, UK). Column cleaning and storage conditions were performed according to manufacturer instructions. All reagents used were of HPLC grade by Sigma Aldrich (USA).

Oxalic acid was separated and quantified using the method previously described by Khaskhali *et al.*, modified as follow:

- ✓ the same mobile phase was used during elution and was composed of a filtered (Minisart® 0.20 μm, Sartorius, Germany) and de-gassed solution of potassium phosphate 20 mM, buffered at pH 3 with orthophosphoric acid;
- ✓ aqueous oxalic acid standards were prepared in the range 0.02–20 mM starting from a fresh stock solution of 200 mM of oxalic acid. These solutions were stable for 3 months at 4° C;
- ✓ prior to analysis, the column was purged by pumping the mobile phase at 4 ml/min for 5 min and equilibrated with the mobile phase at a flow of 0.7 ml/min;
- ✓ the total cycle time was 35 min with a flow of 0.7 ml/min and 20 μl injections from each sample;
- ✓ the detector wavelength was fixed at 210 nm.

3. Results

3.1. Optimal growth conditions of probiotic strains for the assessment of the oxalate-degrading activity

The growth kinetics at 37°C of 13 *Lactobacillus* strains in MRS broth and of 5 *Bifidobacterium* strains in MRS broth supplemented with 0.5 g/l of L-Cysteine hydrochloride were determined, and compared with the same cultures grown in the oxalate supplemented medium SM. For bifidobacteria the incubation was performed under anaerobic conditions.

Lactobacilli presented similar specific growth rated and similar biomass yields (Table 8), suggesting that oxalate does not hamper growth of lactobacilli. Conversely, growth of bifidobacteria was affected by the presence of 10 mM oxalate, since OD600 was generally significantly lower in SM than in MRS ($P < 0.01$, Table 9). Therefore, all subsequent degradation experiments were performed with 10 mM oxalate.

***Lactobacillus* strains**	**Strain**	**MRS medium OD 600 nm 24h MEAN±SD**	**SM medium OD 600 nm 24h MEAN±SD**	**Paired Student's t-Test (p)**
Lactobacillus paracasei	LPC 09	1.75±0.05	1.71±0.04	*
Lactobacillus gasseri	LGS 01	1.80±0.06	1.79±0.04	
Lactobacillus gasseri	LGS 02	1.71±0.05	1.70±0.04	
Lactobacillus acidophilus	LA 07	1.70±0.05	1.70±0.04	
Lactobacillus acidophilus	LA 02	1.78±0.04	1.77±0.04	
Lactobacillus plantarum	LP 01	1.85±0.05	1.83±0.04	
Lactobacillus reuteri	LRE 03	1.82±0.04	1.80±0.04	*
Lactobacillus reuteri	LRE 02	1.60±0.05	1.56±0.04	*
Lactobacillus rhamnosus	LGG	1.41±0.05	1.23±0.04	**
Lactobacillus reuteri	LRE 04	1.36±0.04	1.16±0.04	**
Lactobacillus rhamnosus	LR 06	1.29±0.04	1.21±0.04	*
Lactobacillus rhamnosus	LR 04	1.18±0.03	1.11±0.04	*
L. delbrueckii subsp. *delbrueckii*	LDD 01	0.50±0.03	0.50±0.04	

Table 8. Lactobacillus strains 24h OD values in MRS and SM media (statistical differences were calculated using Student's t Test; * $p<0.05$ ** $p<0.01$)

Bifidobacterium strains	Strain	MRS medium+ CysHCl OD 600 nm 24h MEAN±SD	SM medium OD 600 nm 24h MEAN±SD	Paired Student's t-Test (p)
Bifidobacterium breve	BR 03	1.65±0.04	1.41±0.05	**
Bifidobacterium animalis	DSM20104	1.30±0.03	1.02±0.03	**
Bifidobacterium longum	BL 02	1.03±0.03	0.90±0.03	**
Bifidobacterium longum	BL 03	1.30±0.04	1.10±0.04	**
Bifidobacterium lactis	BA 05	1.10±0.02	0.70±0.02	**

Table 9. Bifidobacterium strains 24h OD values in MRS+Cys•HCl and SM media; growth in MRS+Cys•HCl was higher than in SM medium (statistical differences were calculated using Student's t Test; **p<0.01)

3.2. Oxalate-degrading activity test

Oxalate-degrading activity of different bacterial strains was tested in SM broth, that contained ammonium oxalate 10 mM. At the end of the incubation oxalate concentration was assessed by HPLC. Oxalate degradation (expressed as % removal of the initial oxalate content) is shown in Table 10.

Bacterial species	Strain	Degrading activity (%) MEAN±SD
Oxalobacter formigenes	DSM 4420	98.27±3.9
Lactobacillus paracasei	LPC 09	68.5±5.0
Lactobacillus gasseri	LGS 01	68.4±4.3
Lactobacillus gasseri	LGS 02	66.2±4.1
Lactobacillus acidophilus	LA 07	54.3±5.1
Lactobacillus acidophilus	LA 02	51.4±3.6
Lactobacillus plantarum	LP 01	40.3±3.8
Lactobacillus reuteri	LRE 03	33.9±2.9
Lactobacillus reuteri	LRE 02	31.4±3.4

Bacterial species	Strain	Degrading activity (%) MEAN±SD
Bifidobacterium breve	BR 03	28.2±3.9
Bifidobacterium animalis	DSM20104	27.7±4.2
Bifidobacterium longum	BL 02	27.0±4.3
Bifidobacterium longum	BL 03	25.3±3.8
Lactobacillus rhamnosus	LGG	23.6±2.5
Lactobacillus reuteri	LRE 04	16.8±3.2
Lactobacillus rhamnosus	LR 06	15.7±3.4
Bifidobacterium lactis	BA 05	15.5±3.1
Lactobacillus rhamnosus	LR 04	12.9±4.5
L. delbrueckii subsp. *delbrueckii*	LDD 01	11.3±4.1

Table 10. Oxalate degradation of *Lactobacillus* and *Bifidobacterium* strains after 24h growth in SM medium containing 10 mM oxalate, expressed as the mean ± SD from 3 different experiments.

O. formigenes was used as positive control and almost completely degraded oxalate (> 98%), while potentially probiotic strains exhibited degradation activity ranging from 11.3 to 68.5%. Most lactobacilli were more efficient than *Bifidobacterium* strains in oxalate degradation. Among lactobacilli, the strain exhibiting the highest degradation was *L. paracasei* LPC 09 (DSM 24243) that converted 68.5% of ammonium oxalate, followed by the strains belonging to the species *L. gasseri* and *L. acidophilus*, whose capability to degrade oxalate ranged between 51.4% and 68.4% as shown in Figure 13.

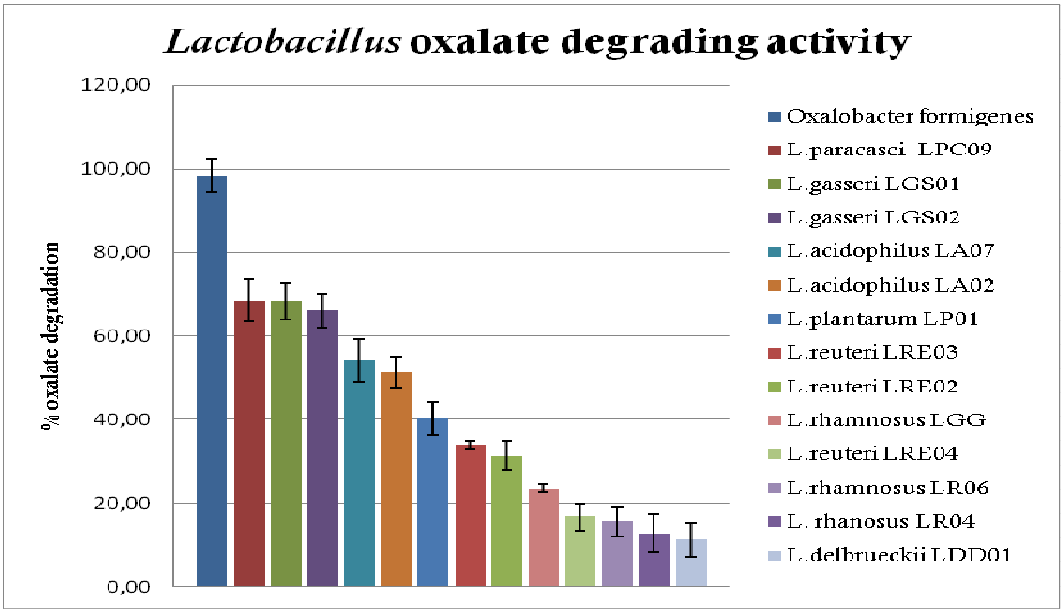


Figure 13. *Lactobacillus* oxalate degrading activity

The oxalate degrading results of Bifidobacteria are shown in Figure 14.

Bifidobacteria performed worse, probably due to intrinsic oxalate toxicity toward this genus. Consistently with this hypothesis, all the bifidobacteria exhibited lower OD_{600} in presence of oxalate than in control MRS cultures ($P < 0.05$).

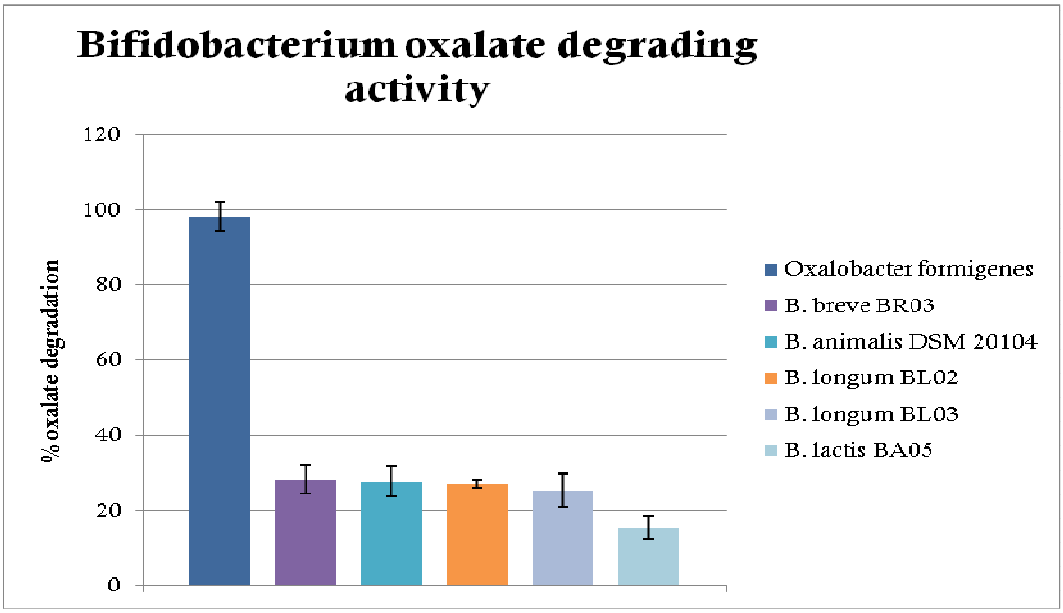


Figure 14. *Bifidobacterium* oxalate degrading activity

4. Discussion

Oxalate can be toxic for bacteria. It is not the primary source of energy for most bacteria, as it has a low energy yield when catabolized. Two major groups of oxalate-degrading bacteria can be identified (Sahin, 2003): the "generalist oxalotrophs", which do not depend entirely on oxalate as an energy source and can ferment many other substrates in addition to it, and the "specialist oxalotrophs", which use oxalate as their sole or major carbon source.

Since oxalate degrading bacteria are present in endogenous microbiota of human intestine, it is possible to hypothesize that the administration of oxalate-degrading bacteria could be an alternative and original approach to reduce the intestinal absorption of oxalate and the resulting urinary excretion. Several studies highlighted the oxalate degrading activity of different *Lactobacillus* and *Bifidobacterium* strains. However, it should be noted that these studies cannot be directly compared quantitatively since there are several variations in the protocols used. For example, different incubation times (1–5 days) and different concentrations of oxalate (range 5–20 mM) were used, a phase in which cells were pre-adapted to oxalate was performed or not, and finally different analytic protocols were applied (enzymatic kits, HPLC or Capillary Electrophoresis).

The general trends of the findings are, however, of interest. Campieri *et al*. (2001) identified potential probiotic strains through evaluating oxalate degradation by pure cultures of *L. acidophilus*, *L. plantarum*, *L. brevis*, *S. thermophilus*, and *B. infantis*. Among lactobacilli, a strain of *L. acidophilus* showed the highest breakdown percentage of 10 mM ammonium oxalate (11.8%), and a strain of *L. brevis* the lowest (0.9%). Weese *et al*. (2004) also reported considerable variation in oxalate degradation by different probiotics *in vitro*. They reported a mean oxalate degradation of 17.7% related to 37 LAB, but they did not further identify the strains. Turroni *et al*., (2010) screened 14 *Bifidobacterium* strains for oxalate degradation activity. Among the tested strains, no oxalate consumption was observed for *B. adolescentis*, *B.*

bifidum, *B. breve*, *B. catenulatum*, *B. longum* biotype *longum*, and *B. longum* biotype *suis*, whereas all the tested *B. animalis* subsp. *lactis* strains proved to be equally active, with 100% oxalate degradation. Moreover, the *oxc* gene was detected by PCR only in the *B. animalis* subsp. *lactis*. Previously, Azcarate-Peril et al., (2006) reported the presence of *frc* and *oxc* genes in *L. gasseri* and *B. lactis.* Turroni *et al.*, (2007) screened the oxalate-degrading ability in 60 *Lactobacillus* strains belonging to 12 species, detecting residual oxalate with an enzymatic assay. Among the tested strains, *L. acidophilus* and *L. gasseri* showed the highest oxalate-degrading activity. Moreover, the presence of *oxc* and *frc* genes, demonstrated by gene-specific PCR, was shown in all *L. acidophilus* and *L. gasseri* isolates that degraded more than 50% oxalate.

In this work, a preliminary screening concerning oxalate degrading activity in 13 *Lactobacillus* strains and 5 *Bifidobacterium* strains, after growth in 10 mM ammonium oxalate medium, was carried out by HPLC analysis. The results obtained suggest considerable variability in the ability of probiotic microorganisms to degrade oxalate *in vitro*. The strain *Lactobacillus paracasei* LPC09 (DSM 24243) showed a marked *in vitro* oxalate degrading activity, converting the 68.54% of ammonium oxalate. The best converters were individuated among the *Lactobacillus* genus, belonging to *L. casei*, *L. gasseri*, and *L. acidophilus* species. In particular, high activity was found in *L. gasseri* LGS01 (DSM 18299) and *L. gasseri* LGS02 (DSM 18300); also *L. acidophilus* LA07 (DSM 24303) and *L. acidophilus* LA02 (DSM 21717) showed remarkable oxalate-degrading activity. Between the *Lactobacillus* genus, the less encouraging results were those related to strains *L. delbrueckii* subsp. *delbrueckii* LDD01 (DSM 22106) and *L. rhamnosus* LR04 (DSM16605) and LR06 (DSM21981).

In contrast, *Bifidobacterium* species have not shown to possess the same ability to degrade oxalate of *Lactobacillus* spp., probably due to sensibility toward ammonium oxalate concentration in the cultural medium.The strains object of this work have been previously studied and the complete taxonomic classification of strains was performed on the basis of biochemical features, species-specific PCR and Pulsed Field Gel Electrophoresis (PFGE).

Furthermore the principal requirements for probiotic strains, such as the ability of the strains to survive in the GUT, were investigated and confirmed. This preliminary *in vitro* study could be the starting point for subsequent *in vivo* trials, using placebo-controlled, double-blind tests. Further investigations will be also needed to completely identify the enzymes able to mediate this *in vitro* oxalate-degrading activity.

In any case, the results of this study could open new perspectives for the utilization of one or more of the analyzed strains in the formulation a food supplement or a functional food aimed at specifically reducing hyperoxaluria for the treatment of kidney stone disease.

INFLUENCE OF CRYOPROTECTANTS ON THE VIABILITY OF FREEZE-DRYED *Lactobacillus acidophilus*

1. Background

1.1. The stability issue

Probiotics, as well as most other active ingredients used in functional foods or food supplements (e.g., vitamins, polyunsaturated fatty acids, vegetable dried extracts), show an unavoidable decay over time. Furthermore, cells need to be alive when they are consumed, then the decay needs to be considered during the shelf-life time, in order to supply the claimed charge of alive bacteria. For this reason, an overdosage is usually applied at manufacturing time, and it is important to consider that decline is significantly accelerated if the recommended storage conditions are not respected. The evaluation of the decay of viable probiotic cells number over time could be very useful to calculate the half-life of each strain, and consequently the overdosage needed at time zero.

The main parameters that affect stability of probiotics, even in an anhydrous formulation, are:

- water (particularly the free and weakly bound fraction);
- temperature;
- concentration of hydrogen ions (pH);
- osmotic pressure;
- mechanical friction;
- oxygen.

Possible damages coming from chemical and physical phenomena during storage of finished products are higher if the physiological status of the bacterial cells at the end of manufacturing process is suboptimal to a certain

extent. Therefore it is important that the manufacturer of probiotics adopts technological measures to reduce any damage to bacterial cells as much as possible.

The viable cell count decay is a function of each formulation. Stability tests must be conducted for each specific finished product in the same storage conditions as its commercial life. Stability studies of probiotics in each finished product by innovative analytical techniques and correlation with accelerated stability tests provide precise indications on the kinetics of decay which can be properly quantified using the half-life parameter. Half-life, also abbreviated as $t_{½}$, is the period of time necessary for the amount of probiotics undergoing decay to decrease by half, in the specific storage conditions.

In general, the decay curves of living microorganisms in most formulations are not linear in a semi-logarithmic graph, then a little difference with respect to a first order decay occurs. As a consequence, there is not the same viable cell count decay in the same period on stability (for example, 3 months). At a certain temperature, in the first three months there is usually a higher decay rate than in the following three, and so on until an equilibrium is attained, in general after one year or little more, when decay rate becomes almost unchanging. Hence, half-life value after one year is only a little lower than half-life value after two years at the same storage temperature.

Between industrial production of probiotic strains and finished product preparation there are several intervening phases, such as freeze-drying, grinding, mixing and, in the case of tablets, compression, which cause various kinds of stress to bacterial cells. For this reason, the highest mortality rate occurs in the first three or six months on stability, when the weakest cells die. If the current general state of art for probiotic finished products is based on half lives generally between 30 and 80 days, innovative technologies are able to guarantee stabilized products with half-life values higher than 300 days at 25°C, thus allowing a 2-year shelf-life at room temperature without the need for the cold chain. However, for the above mentioned reasons, most probiotic products currently sold on the market often do not meet label claims

as concerns, either in terms of number of viable cells at expiration time, or even the taxonomic classification of bacteria (Aureli *et al.*, 2010).

1.2. The biocompatibility issue

Whatever the active ingredient used in the functional food, it is important to avoid any kind of negative interaction between such ingredient and the other active components and excipients of the formulation. In the case of probiotics, there are many compounds that could be toxic to bacteria and considerably reduce the number of viable cells after rehydration of the product.

For example, it is well know that terpenes are very poorly tolerated by most bacteria, including probiotics (Bakkali *et al.*, 2008). Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are widely used as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. For this reason, many flavors or even active ingredients could carry a significant amount of terpenes able to at least partially kill bacteria after rehydration of the powder.

It is therefore crucial to assess the so-called "biocompatibility" between the probiotic strains intended to be used and all the other ingredients of the preparation, which could reasonably differ depending on the health claims ascribed to the product and the dietary supplement typology (capsule, tablet, vial, sachet, oily suspension, confit or other).

1.3. The viability issue

It is fundamental that the probiotic bacteria maintain their viability and health promoting properties during manufacturing process and subsequent storage, in order to guarantee the beneficial effects of probiotic products. As a

result, emphasis is put by manufacturers on development of strategies and technologies to improve productivity, in order to maximize cell survival during drying and storage, and maintain probiotic functionality throughout all the process, and even in the gastrointestinal tract of the consumer (Koop-Hoolihan, 2001; Marteau *et al.*, 2001; Savini *et al.,* 2010). The choice of growth medium for producing commercial amounts of probiotic bacteria is constrained by costs, production yields, and product type (Chavez *et al.*, 2007).

Surveys of probiotic products on the market have revealed common quantitative and qualitative deficiencies, especially regarding labeling and viability of probiotic strains. These observation indicate that probiotic bacteria production technologies, as well as processing and formulation steps applied, are often non-optimal. In order to minimize the storage decay of probiotic cells, stabilizing techniques that guarantee the immobilization of free water, effectively removing it by the solution, are required (Santivarangkna *et al.,* 2008).

1.4. Freeze-drying

Fermentation of probiotic bacteria is usually followed by freeze drying (lyophilization), a process that removes the water from the cell biomass to a level where the product shows significantly increased stability; furthermore this technology offers a range of other benefits:

- ✓ bacteria can be successfully preserved without loss of activity because the product is dried without excessive heating;
- ✓ drying may be extended until the specified moisture level is achieved;
- ✓ freeze dried bacteria present a very high surface area which enables them to be reconstituted very quickly;
- ✓ storage and transportation costs are reduced because of the decrease in volume and weight.

Freeze-drying is a three step linear process:

1. Freezing,
2. Primary Drying,
3. Secondary Drying.

The first stage consists in freezing the product. The nature and the concentration of the solutes present in the solution to be frozen, as well as the freezing rate, determine the structure of the ice matrix, which will in turn determine how the product will dry. Ideally, the ice crystals should be large, wide and contiguous, extending from the product base to the surface, to minimize impedance to vapor flow. The structure dictates the flow of vapor out of the product and therefore the manner of drying: as a basic principle, fast freezing produces small crystals, whereas slow freezing produces larger crystals. In products where the solute will crystallize readily, product freezing will result in a complete mixture of ice and solute crystals. This behavior is termed *eutectic freezing*.

In the case of frozen crystalline materials, such as a salt solution, the lowest temperature in a system in which the residual liquid phase and solid phase are in equilibrium is called the *eutectic point.* Above the eutectic point, ice and solute concentrations persist, and below a mix of ice and solute crystals is produced.

In the large part of products the solute may persist, together with un-freezable water, as an amorphous, non eutectic mix. In the case of amorphous materials, such as sugar solutions, instead of the eutectic point, there is a g*lass transition temperature* (Tg'). At temperatures below the glass transition temperature, the solute phase will exist in a glassy state, which is brittle. At temperatures above the glass transition temperature, the solute will soften and be more flexible, and this is known as the rubbery state.

Once frozen, the material is dried first by a process known as *sublimation*. The product temperature is kept below its critical (eutectic or glass) temperature, while a vacuum is pulled until the pressure/temperature balance is such that the ice sublimes directly into a vapor without melting. If

the balance is not correctly maintained, and the sample temperature exceeds its critical temperature, the product is said to collapse, in the case of amorphous material, or undergoes eutectic melting, in the case of an eutectic material (Mackenzie, 1985). Depending on the severity and manner of collapse, the product may revert entirely to a structure-less sticky residue, or appear completely normal. However collapsed product, even aesthetically acceptable ones, are more difficult to reconstitute and exhibit a reduction in activity. In particular, probiotics can also suffer reduced shelf-life.

Sublimation leads to evaporative cooling which will lower the product temperature. Consequently, to maintain the sample at a constant temperature, heat must be applied to compensate for sublimation cooling. The energy transfer during sublimation is a critical parameter that must be calibrated at best to provide the change of state: if not enough, the process will be too slow, whereas if too much, excess heat input determines the risk of melting or collapsing of the drying material.

When ice is no more present in the product, the secondary drying takes place, a desorption process in which chemically bound water is removed. In contrast to primary drying, which uses low shelf temperatures and a moderate vacuum, desorption drying is facilitated by raising shelf temperatures and reducing chamber pressure to a minimum. The moisture level at this stage may be around 5 - 10%. Depending on the final moisture level required, the time for the secondary drying may be quite long, and the process quite slow.

Every step of the freeze drying process can potentially be dangerous for the probiotic cells. Damage can occur at different levels:

- during cooling for the risk of cold shock;
- during freezing, since the ice forms and the remaining solution concentrate;
- during drying, particularly when sample collapses as drying proceeds.

Reducing temperature during ice formation is the major stress imposed on a cell. The biophysical changes brought about by the transition of water to ice during cooling are the main causes of damage, rather than the low

temperatures *per se.* As ice crystals grow, inevitably firstly in the extracellular medium surrounding the cells under normal cooling conditions, there is an effective osmotic stress, as the solutes surrounding the cells are concentrated into an ever decreasing solvent volume. An increased tonicity, possible aggregation of protein molecules, pH variation, concentration of potentially toxic impurities, disruption of sulfide bonds cause a number of damaging events including changes in ultra-structures of cell membranes, and loss or fusion of membrane bi-layers.

1.5. Bacterial cell cryoprotection

In order to minimize cell mortality, cryoprotectants (CPA) are widely used in the freeze-drying process. CPA are particular additives which can be provided to cells before freezing, and lead to a higher survival after thawing than that obtained in absence (Champagne, 1991; Carvalho, 2004).

There is considerable divergence across the classes of organic molecules that possess CPA activity and their action mechanism. The historically first cryoprotectant agent was glycerol, a small poly-hydroxylated solute with a high solubility in water and a low toxicity during short-term exposure to living cells. It can interact by hydrogen bonding with water and can permeate across the cell membrane, albeit at a relatively slow rate. Cells may tolerate exposure to glycerol in concentrations ranging between 1 and 5 mol/l. In relation to the properties of this molecule, Lovelock developed its theory of the colligative action of CPA, in the early 1950. In this study, because of the well–known molar depression of freezing point associated with mixtures of solutes in solution, he proposed that, at any given temperature below the ice transition, during cooling, the rise in salts, would be ameliorated by the presence of glycerol. This would prevent the attainment of the critical damaging salt concentration, whilst the whole system could be cooled sufficiently to achieve the "glassy matrix" state.

The increasingly high viscosity of glycerol during lowering of temperature is another property that may inhibit or retard ice crystal growth on a kinetic basis. Afterwards, a broad range of solutes, mostly sugars such as sucrose, glucose, lactose, and trehalose, and many small molecular weight solutes, such as amino acids, were investigated for CPA activity, with a broad range of success (Carvalho *et al.*, 2004; Fonseca *et al.*, 2003; Hubalek, 2003; Leslie *et al.*, 1995).

In some cases, under specific conditions, molecules of much larger molecular mass, up to polymers of several thousand Daltons, such as polyvinyl-pyrrolidone and dextran, demonstrated CPA activity. The protection afforded by polymeric CPA has been linked to their non-ideal behavior in high concentrations aqueous solutions. At concentration above 5% w/v, these agents exert appreciable effects on freezing point depression of the system, higher than those predicted from their molar concentration. The high probability of hydrogen bonding between the multitude of hydrophilic side chains of polymers, and their increasing viscosity during freeze concentration of the solution, combine to restrict ice crystal growth on a kinetic basis (Champagne *et al.*, 1996).

The mode of action of solutes during freezing is likely to be multi-factorial, and it has not been comprehensively explained. Permeable cryoprotectants make the cell membrane more plastic, and bind intracellular water, thus preventing excessive dehydration and formation of large ice crystals in the cell. On the contrary, non-permeable cryoprotectants adsorb on the cell surface forming a viscous layer, cause partial efflux of water from the cell, inhibit the growth of ice crystals, and maintain the structure of ice amorphous in the close proximity of the cell.

A marked toxicity, both osmotic and chemical, can be detected if the exposure to CPA is not optimized. The CPA osmotic toxicity can be readily understood since, when added to cells in the required concentrations (1mol/L upwards), CPA cross cell membranes relatively slowly compared to water, so there is a well-documented rapid efflux from the cells, with associated volume collapse. Cells can only tolerate moderate excursions in cell volume, without

significant damage. The nature of chemical toxicities from CPA are inevitably complex, given the ranges of different molecular structures of the commonly used agents.

Although most CPA (neutral solutes and polyols) are relatively innocuous compared to, for example, exposure to equivalent concentrations of salts, in most cases there is an appreciable time and temperature-dependent effect. Attempts have been carried out to avoid these effects, by minimizing the exposure time to CPA before freezing, and by using lower temperatures of exposure.

Bacteria show widely variable survival during freeze-drying. Species and even strains vary intrinsically in their freeze drying robustness. Cell size and form, age and concentration of the culture, stress-response machinery of the cell, and chemical structure of the cell wall (Gram positive strains being more robust than Gram negative) are critical factors. In addition, several external factors such as suspending medium, the nature of CPA, the rate of cooling, and the extent of drying have an impact on bacterial survival during freeze drying.

For the cryoprotection of lactic acid bacteria, a large selection of compounds has been used. They include skim milk with or without supplements such as polyethylene-glycol, dextran, bovine albumine, glycogen, glycerol, disaccharides such as sucrose, lactose, maltose and trehalose, Me_2SO, glycerol, betaine, sodium ascorbate, glutamate, and maltodextrin (Crowe *et al.*, 1988; Patist *et al., 2005;* De Valdez *et al.*, 1983).

2. Materials and Methods

2.2. Reagents

Bacto Peptone, MRS Difco, yeast extract and meat extract were purchased from Becton Dickinson (Buccinasco MI, Italy). Sodium chloride came from Sigma-Aldrich (Milan, Italy). Yeast extract, Tween 80, and glucose were purchased from Merck (Darmstadt, Germany). Bacteriological

agar were supplied by Oxoid (Milan, Italy). NH_4OH, glycerol, sucrose, and $MgSO_4 \cdot 7H_2O$ were purchased from Carlo Erba.

Powder skim milk was obtained from Chimab (Campodarsego, PD, Italy); threalose from Faravelli (Milan, Italy), and maltodextrin from Caldic (Origgio, VA,Italy).

Clindamycin and ciprofloxacin came from Sigma-Aldrich (Milan, Italy). MRS Agar medium and Anaerocult A kits were supplied by Merck (Darmstadt, Germany).

2.2. Production of biomass of *Lactobacillus acidophilus*

Lactobacillus acidophilus NCINB 701748 was kept at -80°C for long term storage, after addition of 20% sucrose and 12.5% glycerol. After thawing, the bacterial strain was grown in MRS broth. In order to reach an optimal bacterial vitality before starting the experiment, the strain was sub-cultured thrice in MRS broth, at 37°C for 24 h.

A "MRS modified" broth was used as fermentative medium for the pilot fermentation, prepared according to the following recipe (g/l):

- glucose 40.00 g
- yeast extract 5.00 g
- MgSO4·7H2O 0.10 g
- meat extract 10.00 g
- Tween 80 1.00 ml
- Distilled water to 1,000 ml

Each ingredient was dissolved in distilled water. pH was measured and corrected to 6.50 ± 0.05, if necessary. The liquid medium was dispensed into 4 liter glass flasks and autoclaved at 121°C for 15 minutes. After sterilisation, pH at room temperature (25°C ± 1°C) was 6.50 ± 0.20.

Eight liters of sterilized cultural broth were transferred to a 10 liter pilot reactor (Figure 15), previously sterilized with steam, and the temperature was setted to 37°C ±0.5. The reactor was inoculated with overnight bacterial culture (2% v/v), stirred at 50 rpm and pH was maintained at 5.50-5.70 by adding NH_4OH 4M as the neutralizing agent. No aeration was use and N_2 was fed to the head space of the reactor at low rate, in order to guarantee anaerobic conditions and to minimize the risk of external contaminations.

The sampling of the culture every 60 minutes was scheduled in the fermentation protocol in order to evaluate the transition between the exponential phase to the stationary phase; optical density (OD) was measured at 560 nm (Figure 16). Additionally, the consumption of the neutralizing agent was monitored throughout fermentation and the stationary phase was considered to be reached when the base addition has slowed down.

Figure15. Pilot reactor

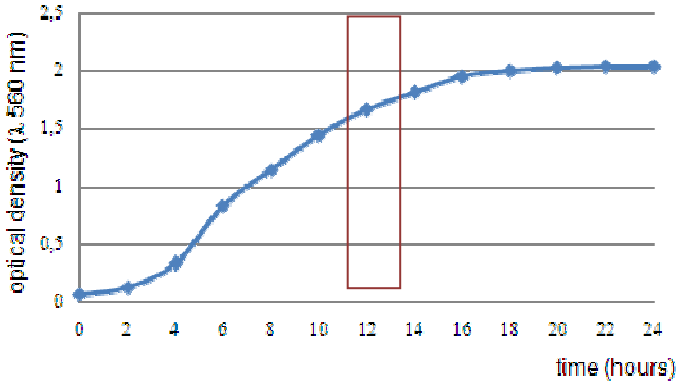


Figure 16. Fermentation curve of *Lactobacillus acidophilus* NCIMB 701748; in red rectangle the early stationary phase.

After fermentation, early stationary phase culture (Figure 16) was harvested (6000 g x 15 min, 20°C). Supernatant was discarded and biomass was washed with sterile water in order to eliminate the residual culture medium.

A variety of cryoprotectant agents were added to bacterial biomass (Table 11). The cryoprotectant solutions were previously autoclaved at 121°C for 15 minutes. For the preparation of the cryoprotected samples to be freeze-dried, a mixture of 80% biomass and 20% cryoprotectant agent (concentrated at 50%w/v) was prepared in order to achieve a final solute concentration of 10% w/v.

After 1 h at room temperature, cells suspensions were frozen at –40°C in glass Petri plates or in glass vials, with a thickness of 1 cm. Thermocouples were placed in the bottom of two different vials to monitor product temperature. The freeze-dried samples were stored in aluminum foil sachets (5 g *per* portion).

Medium code	Cryoprotectant Agents
A	No cryoprotective agent
B	RSM (reconstituted skim milk)
C	Trehalose
D	Sucrose
E	Maltodextrin
F	Trehalose + glycerol
G	Sucrose + glycerol
H	Maltodextrin + glycerol

Table 11. Composition of cryoprotectant agents

2.3. Freeze-drying parameters

Freeze-drying was performed in a Edwards pilot plant.Two different freeze-drying cycles have been used and compared:

1) Cycle at low freezing temperature (**LT**):

–40°C to + 25°C at 0.12 mbar pressure in 48 h, followed by 15 h at 0.01 mbar.

2) Cycle at high freezing temperature (**HT**):

–20°C to +25°C at 0.12 mbar pressure in 30 h, followed by 15 h at 0.01 mbar.

2.4. Enumeration of viable cells

Lactobacillus acidophilus viable cells were enumerated using the method of plate colony-count. Initial suspension of samples and decimal dilutions in peptone saline solution were performed according to ISO 6887-5:2010.

The peptone saline solution used had the following composition (g/l):

- Bacto Peptone (enzymatic digest of animal proteins) 1.00 g
- Sodium chloride 8.50 g
- Distilled water to 1,000 ml

Each component was dissolved in distilled water. pH was measured and corrected to 7.00 ± 0.05, if necessary. The solution was dispensed into glass bottles and autoclaved at 121°C for 15 minutes. After sterilisation, pH at room temperature (25°C ± 1°C) was 7.00 ± 0.20.

The count of *Lactobacillus acidophilus* was performed using MRS Agar medium supplemented with 0.1 µg/ml clindamycin and 10 µg/ml ciprofloxacin (MRS/CL/CIP agar, ref. ISO 20128:2006/ IDF 192:2006). MRS Agar medium supplemented with clindamycin and ciprofloxacin was prepared according to the MRS broth recipe reported in Section 2.1., pag.45. In addition, 15.0 grams of bacteriological agar were added to 1 liter of medium and completely dissolved in a water bath at 80°C. The hot medium was dispensed into glass bottles and autoclaved at 121°C for 15 minutes.

At the moment of use, the following ingredients were added to the basal medium after melting and thermostating in a water bath to 45°C ± 1°C:

- 0.05 ml of a clindamycin stock solution (0.2 mg/ml), sterilised by 0.22 µm filtration, to 100 ml of MRS agar, in order to obtain a final clindamycin concentration equal to 0.1 µg/ml;
- 0.5 ml of a ciprofloxacin stock solution (2 mg/ml), sterilised by 0.22 µm filtration, to 100 ml of MRS agar to obtain a final concentration equal to 10 µg/ml.

A volume of 0.1 ml of the appropriate dilutions (10^{-8}, 10^{-9} and 10^{-10}) was transferred onto the surface of two Petri dishes containing the MRS/CL/CIP agar and the sample was spread over the entire surface of the medium using a sterile spreader. All plates were incubated for 48 to 72 hours at 37°C under anaerobic conditions (GasPak system) with Anaerocult A.

After the defined incubation time, the dishes having a number of colonies between 10 and 300 CFU were counted; the result was expressed as colony forming units (CFU) per gram, using the following formula:

$$\frac{\Sigma\, c}{(n1 + 0.1\, n2)\, d}$$

where:

$\Sigma\, c$ is the sum of colonies counted on all dishes retained

$n1$ is the number of dishes retained in the first dilution

$n2$ is the number of dishes retained in the second dilution

d is the dilution factor corresponding to the first dilution retained

All analysis were made in triplicate.

2.5. Water Activity Determination

Water activity is a dimensionless quantity used to represent the energy status of the water in a system. It is defined as the vapor pressure of water above a sample, divided by that of pure water at the same temperature; therefore, pure distilled water has a water activity of exactly one. It is widely used in food science and pharmaceutical production as a simple, straightforward measure of the dryness of the products, and it's directly connected to the shelf life of the products.

Free water means that part of water molecules not bound to sugars, starch, pectins and therefore immediately available for the microbial metabolism.

The analytical method requires the use of a instrument (Aqualab-(Model Series 3 TE, Decagon Devices Inc.), which measures the free water contained in a sample, using the technique of the dew point on a chilled mirror.

This technique consists in taking to equilibrium the sample with the head space of a hermetically locked chamber, containing a mirror and sensors for the measure of the dew on it.

In a equilibrium state, the relative humidity of the air in the chamber is the same of the water activity in the sample.

In Aqualab the mirror temperature is controlled with precision by a thermoelectric refrigerator (Peltier). The detection of the exact moment when the first condensation occurs on the mirror is observed by a photocell.

A beam of light is directed onto the mirror and reflected in a photoelectric detector; this detects a change in reflection when the condensation occurs on the mirror. A thermocouple fixed on the mirror registers the condensation temperature and the water activity and the sample temperature are shown on the display.

The fan is to speed equilibrium and to control the boundary layer conductance of the dew-point sensor.

For variation of one degree of temperature respect to the reference of 23°C, a variation of ± 1/°K occurs, that means ± (1/273+23) = ± 0.003. This calculation does not take into account the characteristics of adsorption isotherm of the product.

The reference Methods for water activity determinations are detailed in the Official Methods of Analysis of AOAC International,1995.

2.6. Statistical analysis

Statistical significance of the parameter differences was evaluated by t-test method. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Growth curve of *Lactobacillus acidophilus* NCIMB 701748

The effect of diverse cryoprotectant media on the survival of *Lactobacillus acidophilus* NCIMB 701748 during the freeze-drying process was investigated. Pilot fermentation processes were carried out in order to monitor the growth and to produce enough biomass for the freeze-drying tests (Figure 17).

The early stationary phase was identified monitoring the turbidity of the culture at 560 nm. Stationary phase was reached after 16 h of culture at 37°C. At this time-point, biomass was harvested and mixed to the diverse cryoprotectants.

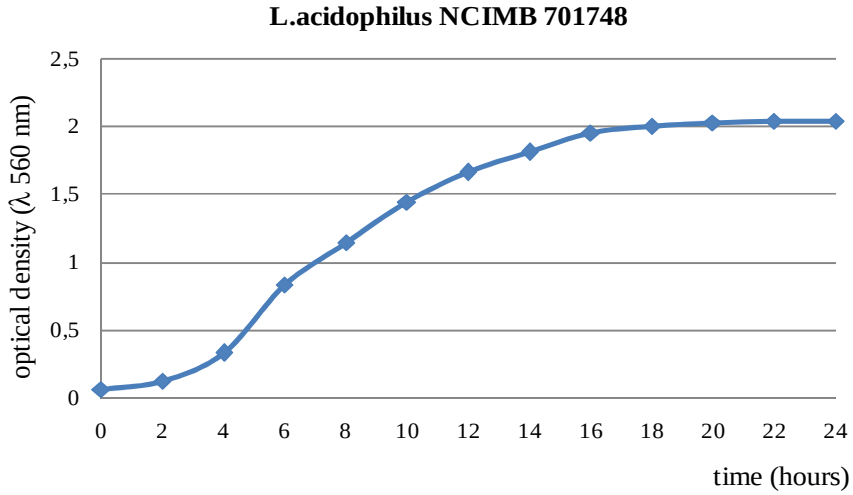


Figure 17. Growth curve of *L. acidophilus* NCIMB 701748. Bacterial strain was grown in MRS modified broth at 37°C in anaerobic conditions.

3.2. Toxicity of cryoprotectans

To evaluate the potential toxicity of the solutes used in the different cryoprotectant media, three different experiments were performed. The cryoprotectant solutions were added to the biomass for an hour at room temperature at the concentrations described in Table 12, then cell survival was evaluated by plate counting. Percentages of cell survival were calculated as

the ratio between the values of viable cell counts after 1 h of contact with the different compounds, and before contact. Cell survival ranged between 92.6 and 96.4% in the tested condition, without any significant difference among the different soultions (Table 12; $P > 0.05$). Therefore, the toxicity of all the cryoprotectants was considered negligible.

Medium code	Solute concentration in the cryoprotected biomass	CFU/ml (T_0)	CFU/ml (1h contact)	% survival after contact
A	No cryoprotective agent (negative control)	35 x 10^9	33x 10^9	94.3
B	5% RSM (positive control)	33 x 10^9	31x 10^9	93.9
C	10% Trehalose	28 x 10^9	26x 10^9	92.8
D	10% Sucrose	27 x 10^9	25x 10^9	92.6
E	10% Maltodextrin	28 x 10^9	27x 10^9	96.4
F	10% Trehalose + 1% glycerol	27 x 10^9	25x 10^9	92.6
G	10% Sucrose + 1% glycerol	28 x 10^9	26x 10^9	92.8
H	10% Maltodextrin + 1% glycerol	27 x 10^9	26x 10^9	96.3

Table 12. Percentages of cell survival in presence of different cryoprotectant solutions. Means of 3 experiments are reported, SD always < 7%.

3.3. Freeze-drying survival

To evaluate cell survival during freeze-drying process, three independent experiments were performed using the low-temperature freeze drying cycle (LT), at freezing temperature of -40°C. The survival of the 3 batches of samples freeze dried with different cryoprotectant is shown in Table 13. Percentages of cell survival have been calculated as the ratio between the total cell population after and before freeze drying process.

Medium code	Solute concentration in the cryoprotected biomass	Survival ratio %			
		I batch	II batch	III batch	MEAN ± SD
A	No cryoprotective agent (negative control)	5.3	8.2	10.9	8.1 ± 2.8
B	5% RSM (positive control)	25.6	29.5	28.5	27.9 ± 2.1
C	10% Trehalose	35.4	42.6	47.6	41.8 ± 6.2
D	10% Sucrose	33.3	42.0	45.3	40.2 ± 6.2
E	10% Maltodextrin	48.3	58.5	64.5	57.1 ± 8.2
F	10% Trehalose + 1% glycerol	38.3	45.8	48.5	44.2 ± 5.3
G	10% Sucrose + 1% glycerol	42.2	44.2	46.2	44.2 ± 2.0
H	10% Maltodextrin + 1% glycerol	50.2	62.2	67.3	59.9 ± 8.8

Table 13. Freeze-drying cell survival. Mean ± standard deviation (SD) of three independent experiments are reported.

If cryoprotective agents were not added (negative control, A) *L. acidophilus* NCIMB 701748 exhibited a 8.1% survival after the freeze-drying process. Positive controls, suspended in 5.0% RSM (reconstituted skim milk), exhibited a 27.9% survival. All the cryoprotectants induced a higher cell survival, compared with both negative (A, $p < 0.01$) and positive (B, $p < 0.05$) controls (Figure 18).

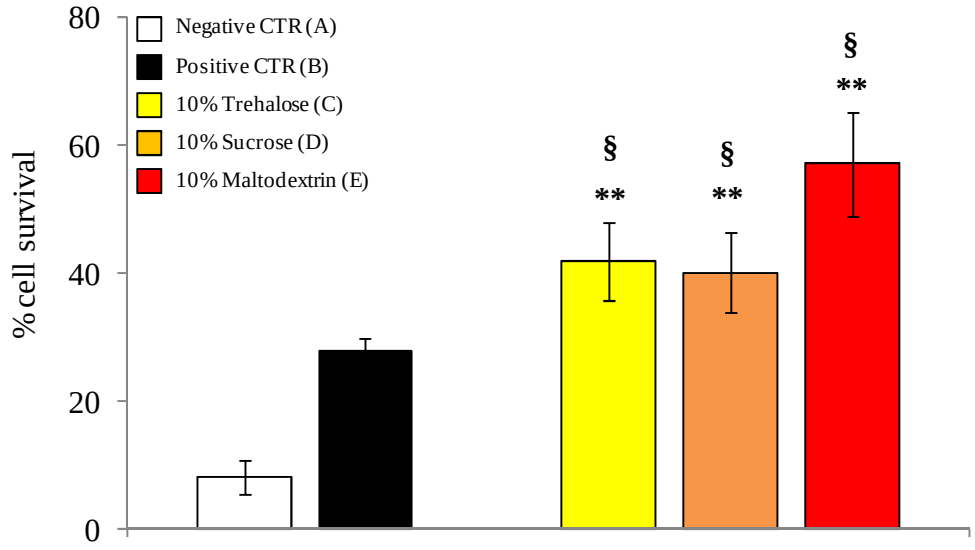


Figure 18. Percentage of cell survival of samples added with 10% Trehalose (C), 10% Sucrose (D) and 10% Maltodextrin (E). Statistical differences were calculated using student's t-test (** $p < 0.01$ vs negative control; § $p < 0.05$ vs positive control).

The survival of *L. acidophilus* NCIMB 701748 was slightly higher if 1% glycerol was added to the cryoprotectants (Figure 19), but difference was statistically significant only with maltodextrin.

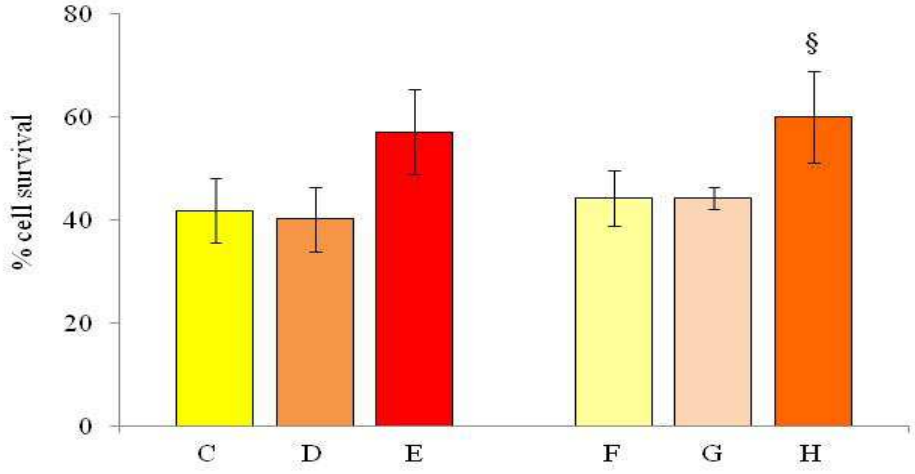


Figure 19. Percentage of cell survival of samples added with 10% trehalose (C), 10% Sucrose(D) and 10% Maltodextrin (E), 10% Trehalose plus 1% glycerol (F), 10% Sucrose plus 1% glycerol (G) and 10% Maltodextrin plus 1% glycerol (H). Statistical differences were calculated using Student's t-test (§ $p < 0.05$ versus E sample).

Furthermore, the presence of glycerol caused water activity (aw) to increase, leading to potentially critical values in aw all the samples supplemented with glycerol (Figure 20).

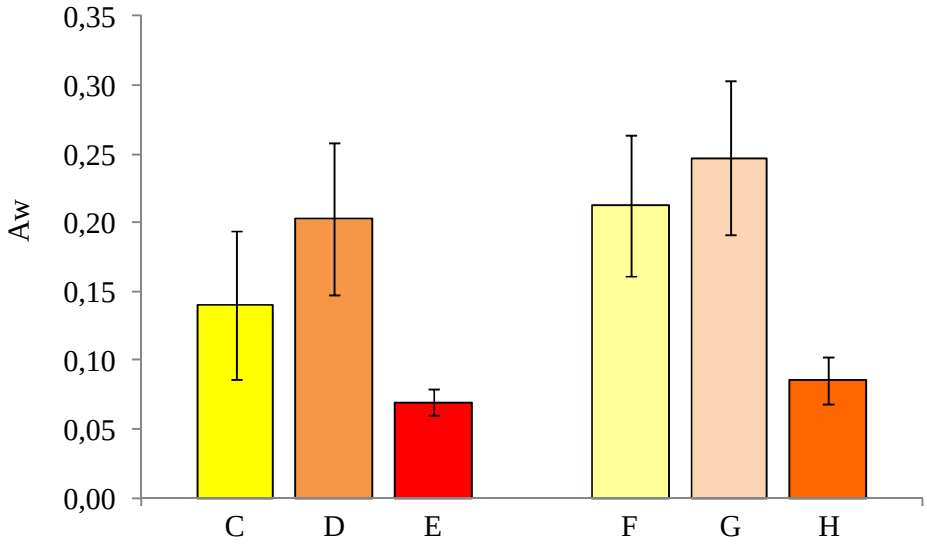


Figure 20. Aw of samples supplemented with 10% Trehalose (C), 10% Sucrose (D) and 10% Maltodextrin (E), 10% Trehalose plus 1% glycerol (F), 10% Sucrose plus 1% glycerol (G) and 10% Maltodextrin plus 1% glycerol (H). No statistical differences have been found ($p > 0.05$).

Physical stability of freeze-dried bacteria is extremely dependent on water activity. Dried materials tend to come to a final equilibrium with their environment. If the relative humidity of the environment is lower than water activity of the product at the equilibrium, the last will release water until it reaches equilibrium with the environment. Freeze-dried probiotic cells having an initial aw value of 0.2 could suffer low stability problems, in the long term shelf–life of the final designed formulations.

In figure 21 is shown the physical structure of 8 freeze dried samples obtained with the LT lyophilization cycle at -40°C. The appearance and the structure of the cakes obtained with LT cycle are acceptable in all cases; no signs of collapse have been pointed out.

Figure 21. Pictures of the eight samples obtained with cycle LT

Cryoprotectants trehalose (C), sucrose (D), or maltodextrin (E) were selected for further investigations, whereas glycerol-supplemented cryoprotectants were no longer investigated because of the higher values in aw, potentially critical in the long term storage of the products. They were utilized as cryoprotectants in a high-temperature freeze drying cycle (HT), carried out at higher freezing temperature -20°C. The purpose of this experiment was to compare the three carbohydrates C, D, and E, which exhibit different glass transition temperature (Tg'), in this more critical freeze drying cycle. In fact, the Tg' value of trehalose and sucrose solutions is lower than the freezing temperature, while the Tg' value of maltodextrin solution is higher (Table 14).

Medium code	Solute concentration in the cryoprotected biomass	Solute Tg'	% Survival
A	No cryoprotectant agent	//	7.55
B	5% RSM	-12°C	23.15
C	10% Trehalose	-29.5°C	44.25
D	10% Sucrose	-32°C	48.36
E	10% Maltodextrin	-11°C	55.8

Table 14. Tg' of CPA and survival rate

Likewise in the LT cycle, samples with cryoprotectant agents C, D, and E exhibited significantly higher survival ($P < 0.05$) than controls A and B (Table 14; Figure 22), and maltodextrin was confirmed the most effective cryoprotectant in terms of survival ratio.

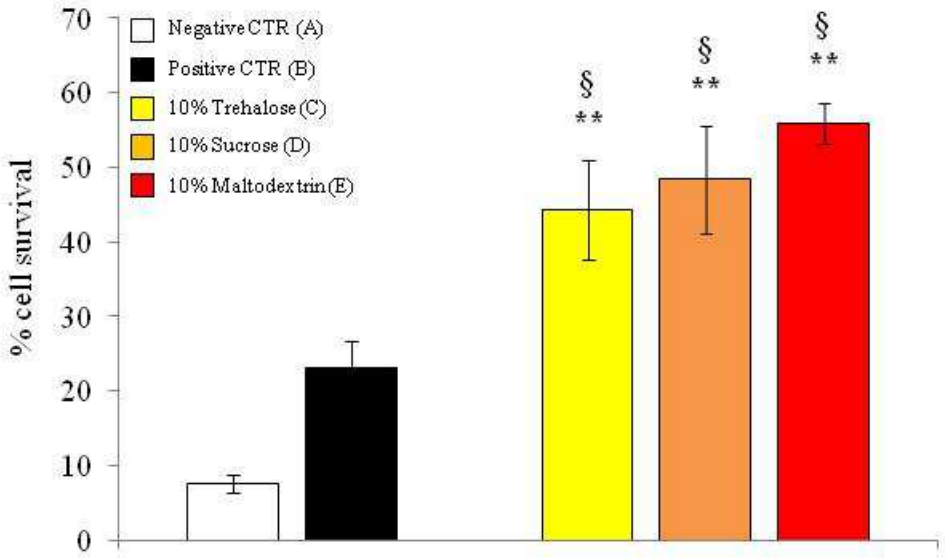


Figure 22. Percentage of cell survival of samples added with 10% Trehalose (C), 10% Sucrose (D) in HT cycle. Statistical differences were calculated using Student t-test (** $p < 0.01$ vs negative control; § $p < 0.05$ vs positive control).

As shown in figure 23, the parameters of the HT freeze-drying cycle lead to products with very different structures.

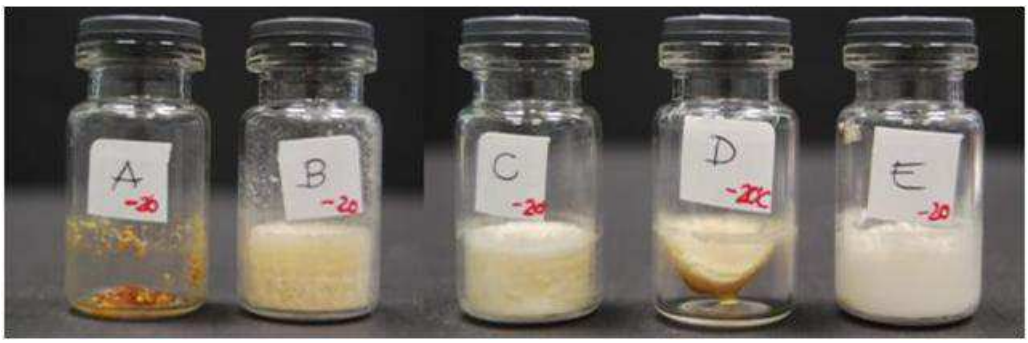


Figure 23. Pictures of the 5 freeze-dried samples using HT cycle.

When primary drying is conducted at temperatures > Tg', the mobile water present that the interstitial region can no longer maintain its original configuration, and a condition referred to as collapse occurs. The absence of cryoprotectants leads to a completely collapsed product (sample A). Samples B and C present a good structure, while D has suffered a slight collapse during primary drying, resulting in shrinkage; sample E presents the best structure.

4. Discussion

There are many technological challenges in producing probiotic cultures. From a culture's supplier perspective, key targets include culturing the microorganism to high cell concentration, minimizing cell death during freeze drying, and maintaining the functional properties of the cells during the production process and storage. Probiotics in freeze dried form should have an acceptable cake structure, good rehydration time and retention of active viability with sufficient stability at the required storage temperature.

One of the aims of this work was to evaluate the protective effect of trehalose, sucrose, and maltodextrin with or without glycerol in the freeze-drying step of *L. acidophilus* NCIMB 701748, and to compare two freeze-drying cycles in order to select the most efficient in terms of strain viability and economical aspects of the process. This specific strain was selected for its intrinsic sensitivity to freeze drying and storage. The first assessment concerned the toxicity of the cryoprotectants (CPA) selected for the study, carried out comparing *L. acidophilus* viable cell counts before and after addition of various CPA. This comparison showed a generally very low toxicity versus the strain in the conditions considered (1 hour at room temperature). The conclusion was that every solute tested can be considered compatible with the bacterial cells at the tested dosages.

Recovery rate of *L. acidophilus* culture after freeze drying in the absence of CPA (negative control, A) was absolutely scarce (about 7.5%) in a

producer's view. Reconstituted skim milk (positive control, B) is a commonly recognized effective cryoprotectant medium, because of the protective action of milk proteins for the cells. However, results of survival rate of sample B are worse than those obtained with the other CPA tested in this study at both freeze-drying cycles used. Disaccharides, such as trehalose and sucrose (samples C and D), are not permeable CPA. Their protective activity might be due to the ability to prevent injurious eutectic freezing of cell fluids by trapping salts in a highly viscous or glass-like phase. These carbohydrates could help to prevent or decrease the lethal effect of intracellular ice formation during freezing, through hydrogen binding with water and cell structures, or may protect against free radicals produced during storage and rehydration of freeze-dried cultures. The results of this work show that both trehalose and sucrose enhanced the retention of viability of *L. acidophilus* in freeze-drying step, even compared to positive control B.

Maltodextrin, a polymer of D-glucose with a high molecular weight, exerts a protective action in freezing, increasing the viscosity of the extracellular medium. Moreover, the high glass transition temperature gives an additional advantage in freeze-drying process, because the risk of structure collapse is very limited. The addition of glycerol, a polyalcohol that can slowly penetrate the cells, modifying the rate of crystal growth and membrane permeability, enhanced slightly the viability of the samples prepared with the carbohydrate based CPAs (samples F, G and H). However, the free water (aw) of freeze dried samples in presence of glycerol resulted considerably higher than the corresponding without glycerol, and it is well known that this parameter could represent a risk for shelf-life, enhancing the bacterial metabolism during storage. The results of the present study indicates that the disaccharides trehalose and sucrose improve the retention of the viability of *L. acidophilus*, when low temperature freeze-drying cycle is applied, although maltodextrin has proved to be more efficient CPA in relation to survival rate and aw.

During the high temperature freeze-drying cycle inevitable problems of collapse occurred to sample D, having a Tg' lower than freezing temperature.

Cake of sample C maintained a cosmetically acceptable appearance, albeit the presumable micro-collapse during primary drying could cause a poor rehydration time and low retention of viability. Values in survival rate and aw showed that maltodextrin results the more effective CPA used even in HT cycle. Furthermore, the resulting cake has a robust structure without any signs of collapse. The previous results point out the role of this molecule presenting high collapse temperature, and the interest of using it as a bulking agent for freeze drying applications.

Freeze-drying is a relatively expensive process. The equipment is about three times as expensive as the equipment used for other separation processes, and the high energy demands lead to high energy costs. Furthermore, freeze drying has a long process time, because the addition of too much heat to the material can cause melting or structural deformations. From a practical point of view, it is clearly admitted that controlling the heat and mass transfers is of great importance for the optimization of the freeze-drying process. The physical characteristics of maltodextrin allows an increase in the product temperature during the primary drying stage, decreasing significantly the duration of the cycle and the amount of energy transferred. A 1°C increase in shelf temperature during primary drying can lead to a 13% decrease in drying time. Therefore the choice of the most effective cryoprotectant agent can lead to a significant saving of both time and money, whilst still retaining a good end product.

CONCLUSIONS

It is well known that diet plays an important role in the maintenance and improvement of human health through the provision of growth substances for the microbiota. To generalize, it is possible to categorise the gut microbiota components on the basis of whether they exert potentially pathogenic or health-promoting effects. Lactic acid producing genera such as bifidobacteria and lactobacilli have along standing "health image". As such, attempts to stimulate microorganisms that carry out the latter, could give many benefits.

Attention has turned towards food materials that may offer improved health benefits. The so-called "functional foods" have a currently high profile and many new products exist or are being developed. Early approaches involved vitamin or mineral supplementation to the diet. However, recent developments have focused on gut functionality which is a realistic target for enhanced food ingredients. Using diet to prophylactically manage the gut microbiota is both user friendly and attractive to the consumer.

One of the most noticeable and best acknowledged approach for modulating gut microbiota composition is the supplementation of probiotics strains, that is alive bacteria able to exert many beneficial effects on human health. In particular, the selection of probiotic strains that present specific physiological features represents a novel frontier and can be exploited to develop properly targeted strains. In this perspective, the Selenium and Zinc internalization by two probiotic strains, *Lactobacillus buchneri* Lb26 (DSM 16341) and *Bifidobacterium lactis* Bb1 (DSM 17850) has been studied, and the bioavailability of the probiotic minerals has been evaluated. A Caco-2 cell line was purposely employed to resemble human colonic cells and to determine the adsoption and transportation of different forms of Se and Zn (probiotic, organic, and inorganic). The different tested compounds result to be transported at considerably different extents across the Caco-2 cell monolayer, suggesting a higher *in vivo* bioavailability of the probiotic forms compared to the inorganic and even the organic molecules used. The Se and

Zn forms internalized by *L. buchneri* Lb26 (DSM 16341) and *B. lactis* Bb1 (DSM 17850), respectively, showed the highest absorption and cell diffusion across Caco-2 cells, therefore suggesting a notably improved bioavailability and systemic relevance.

In order to develop oxalate-degrading probiotic strains, an *in vitro* screening of a variety of bifidobacteria or lactobacilli with regard to their oxalate-degrading activity has been carried out. These strains can be potentially useful for human health, because humans lack the enzymes needed to metabolize endogenous and dietary oxalate, a toxic compound causing hyperoxalutia and calcium oxalate urolithiasis.

Oxalate degradation resulted both species- and strain-specific. The strains showing the major degrading activity were *Lactobacillus paracasei* LPC09 (DSM 24243) and, among the *Lactobacillus* genus, the species *L. casei*, *L. gasseri*, and *L. acidophilus*. Conversely, the less encouraging results were obtained with *L. delbrueckii* subsp. *delbrueckii* LDD01(DSM 22106) and with the two *L. rhamnosus* strains. In contrast, bifidobacteria did not present an oxalate degradation capability comparable with that of *Lactobacillus* spp., probably because of the sensibility toward ammonium oxalate concentration in the cultural medium or of the lack of pre-adaption to oxalate before incubation.

Another research topic focused on the ability of certain molecules to effectively protect and improve viability of probiotic cells during freeze-drying, a common process used to preserve bacteria for both food and pharmaceuticals applications. It is well known that the stability of probiotic bacteria during dehydration process and subsequent storage can be greatly improved using protective agents. Dehydration can lead to irreversible changes in bacteria cells, such as changes in the structure of sensitive cell proteins and membrane lipids, resulting in loss of cell viability. One of the goals of this study was also the comparison of the performances of three cryo-protective agents in two different freeze-drying processes, with very different initial temperature values.

The disaccharides trealose and sucrose and the polymer maltodextrin are able to enhance the retention of viability of *L. acidophilus* NCIMB 701748 in freeze-drying process. The addition of glycerol to the cryo-protective agents tested, enhanced slightly viability. However the supplementation of glycerol determined an increase in free water (aw) of freeze dried samples, that can be a risk for shelf life, enhancing the bacterial metabolism during storage.

The disaccharides trehalose and sucrose improved the retention of the viability of *L. acidophilus*, when low temperature freeze-drying cycle was applied. Maltodextrin resulted the most effective cryoprotective agent when cycles performed at higher temperatures were applied. Furthermore, in these cases, the resulting cake presented a robust structure without any signs of collapse. The choice of the most effective cryoprotectant agent can lead to a significant saving of both time and money, whilst still retaining a good end product.

In conclusion, the overall activities of this work contributed to characterize some precise features of probiotic strains that could represent the rationale supporting their use in the multifaceted world of nutraceuticals, specifically finding application in the field of food supplements and functional foods as well. Furthermore, the improvement of freeze-drying technology represents a major target for probiotic production and distribution.

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