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Characterisation of probiotic strains for the control and prevention of enteropathogens in the food chain.

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PART I

INTRODUCTION

Chapter 1. The intestinal microbiota

The term "microbiota" defines the microbial flora harboured by normal, healthy individuals.

The autochthonous microbiota (or indigenous microbiota, or normal microbiota) is a population of different microorganisms in a dinamic equilibrium. This population is formed by resident microrganisms present in all communities of different animal species. They are always present in the gastrointestinal (GI) tract of healthy individuals, they can colonize particular niches in GI and can grow without oxygen in this ecosystem (Berg, 1996; Klaenhammer, 2001). The allochthonous microbiota is a group of microrganisms acquired temporarily and that therefore is unable to colonize a particular habitat, except under abnormal conditions (Berg, 1996). True pathogens are microorganisms acquired accidentally and therefore not normally present in all members of a community of an animal species (Berg, 1996). If persistent, they cause infections and diseases.

According to the *Oxford English Dictionary*, to colonize is "to establish a settlement in a new country" This is precisely what microbes do when they colonize the intestine. Although in most cases the term colonization implies a long-term presence in the intestine, actually this is not always true. The presence of any of the microbes at one particularl time in a particular niche does not necessarily mean that this has been colonized.

At genus level, the intestinal microbiota of an adult appears to be particularly stable, and even at species level, variations in the composition of the microbiota appear to be minor. At strain level, however, a considerable variation in the composition of the faecal microbiota has been observed. Thus, some subjects may be continuosly colonized with new microbes (Isolauri et al., 2004). In a stable GI ecosystem, all available habitats or niches are occupied by indigenous microorganisms. Any

transient species derived from food, water or others (even derived from another part of the GI system or from the skin), often will not adhere and will pass trough the GI tract.

Before birth, the fetus, including its intestine, is sterile. Birth brings about an immediate end to this sterility: microbial colonization begins after birth. The first microbial population the newborn comes into contact with are the maternal intestinal and vaginal microflora. Successively, the newborn will be exposed to the microbes from the environment. The microbial population of the newborn changes from the weaning time to the adulthood, also in relation to other factors like diet (breast versus formula feeding) and country (Berg, 1996; Guarnier and Malagelada, 2003).

The intestinal microbiota of humans and animals is a specific ecosystem made of a complex collection of microorganisms (~ 10^{14} – 10^{15}) which forms an individual microbiota typical for each being . In particular, the human intestinal microbiota consists of more than 400 different species. In 1994, Hagiage divided the intestinal microbiota into two groups: a dominant group and a subdominant group.

- The "dominant microbiota" in human colon is constituted by Gram negative or not sporulating microrganisms principally belonging to the genus *Bacteroides* (Hagiage, 1994) and by Gram positive bacteria from the genera *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus*, *Ruminococcus*, *Veillonella*, *Clostridium*. The concentration levels are 10^{9}-10^{11} ufc per faecal gram with inter-individual modifications (Hagiage, 1994).

The "subdominant group" is constituted by anaerobes and aerobes bacteria, principally belonging to the genera *Enterobacter, Streptococcus and Lactobacillus*. The concentration of this group is lower than that of the dominant group, since it ranges between 10^{6} and 10^{8} ufc per gram or mL of intestinal content (Hagiage, 1994).

The allochthonous microbiota, consisting of variable microbiota among resident microbes, has a concentration of 10^{5}-10^{6} ufc/gr and it includes the following genera: *Citrobacter, Klebsiella, Proteus, Enterobacter, Pseudomonas* and *Staphylococcus* and some yeasts like *Candida albicans*.

The indigenous bacteria are not randomly distributed throughout the GI tract but are found at characteristic levels in particular regions of the tract (Berg, 1996). The oral cavity harbours a complex microbiota of about 200 species, consisting of both strict and facultative anaerobes, like streptococci, lactobacilli, bifidobacteria, *Bacteroides* and yeast, like *Candida albicans*.

Most oral bacteria are destroyed by gastric acid leaving only a few surviving species, so that the gastric concentration usually is less than 10^3 colony forming units/ml (cfu/ml). The small quantity of microbes in this area results also from the peristaltic movement from the stomach through the small bowel (Berg, 1996). The microflora of the stomach is gram-positive and aerobic and the most commonly isolated species are acid-tolerant lactobacilli and streptococci, staphylococci, various fungi, which, unlike the majority of the microorganisms found in food, survive the passage through the stomach.

The small intestine can be considered a zone of transition between the sparsely populated stomach and the luxuriant bacterial flora of the colon. Under normal conditions the microflora of the proximal small bowel (duodenum and jejunum) is similar to that of the stomach. The bacterial concentration is 10^3-10^5 cfu/ml of gastrointestinal contents and the predominant species include streptococci, staphylococci and lactobacilli. Veillonellae and *Actinomyces* sp. are also frequently isolated whereas coliforms and other anaerobic bacteria are found in lower concentrations.

Also the distal small intestine (ileum) is considered a “transition zone”. The microrganism content ranges from the relatively rare microbiota of upper bowel to the rich and diverse microbiota of the large intestine (Berg, 1996). In the distal ileum gram-negative bacteria start to outnumber gram-positive organisms. Coliforms are consistently present, and anaerobic bacteria such as *Bacteroides*, *Bifidobacterium*, *Fusobacterium* and *Clostridium* are found in substantial concentrations. The ability of the distal ileum to support an anaerobic bacterial flora can be seen in its oxidation-

reduction potential (Eh) of -150mV, similar to but not as reduced as the -200mV potential found in the cecum.

Distal to the ileocecal sphincter bacterial concentrations increase sharply. Within the colon the bacterial concentration is 10^{11} to 10^{12} ucf/gr of intestinal contents, consequently the colon is the primary site of microbial colonization in humans and animals, because of its very low oxidation-reduction potential and the slow intestinal motility. Anaerobic bacteria outnumber aerobes by 1000-fold. The predominant ones are *Bacteroides*, *Bifidobacterium* and *Eubacterium*. Anaerobic gram-positive cocci, Clostridia, enterococci and various species of *Enterobacteriaceae* are also common (Fig. 1).

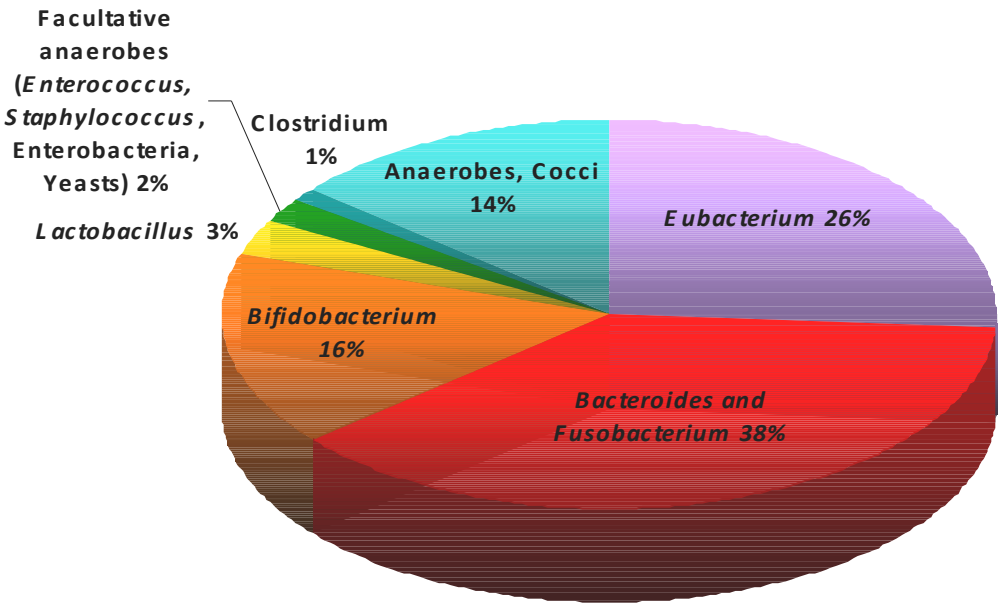


Fig. 1. Composition of human colonic microflora

There are approximately 400-500 species present as indigenous GI bacteria. About 40% of those species have not been cultured in laboratory (Berg, 1996). Researches using molecular procedures have show that many DNA sequences correspond to undescribed microorganisms.

Due to regulatory factors, the colonic microbiota appears stable with a lower cell division due to high mortality, a competition for substrates and the existence of inhibitory factors associated with the release of antimicrobial substances (Hagiage, 1994). The vast majority of human colonic microrganisms are anaerobes. However, they show varying degrees of tolerance toward oxygen, ranging from the relatively oxygen tolerant bifidobacteria and bacteroides to very strictly anaerobic mathanogenic Archeae. These latter outnumber aerobic species by a ratio between 100 and 1000, and the *Bacteroides fragilis* group, bifidobacteria, eubacteria, and a variety of anaerobic Gram positive cocci have proven prevalent. While superficially similar to the faecal microflora in the other animal species, the human microbiota differs in many respects, e.g.. the relatively high numbers of clostridia and enterobacteria.

Only generic information is available about the metabolic relationships that exist between individual bacterial communities in the colon, or about the ecology and multicellular organisation of the microbiota. A variety of host, microbiological and environmental factors affects colonisation of the large bowel (Table 1). The diversity of bacterial species largely derives from the multiplicity of different carbon and energy sources available for fermentation in the colon, and the principal host factors regulating the microbiota are substrate availability and colonic transit time. Various types of ecological interaction take place between intestinal microrganisms, i.e. commensalism, in which one species is stimulated by another, which in its turn is not influenced by the growth and activities of the first; neutralism, with co-existing bacterial communities which however do not have a significant metabolic effect on one another; antagonism, in which the growth of one population is hindered by the inhibitory substances produced by another, and symbiosis, where two species have a necessary dependence on each other. However, the ability to compete for limited nutrients and, in some circumstances, adhesion sites on food particles, colonic mucus or the mucosa, is considered the most important microbiological factor determining

the composition of the microbiota, with unsuccessful species being rapidly eliminated from the ecosystem.

Table 1. Factors affecting bacterial colonisation in the large intestine

Environmental
Amounts and types of substrate available
pH of intestinal contents
Redox potential
Geographical residence/cultural factors associated with host
Host
Diet
Colonic transit time, epithelial cell turnover rates
Disease, drugs, antibiotic therapy, rates of mucus production and its chemical composition, pancreatic and other secretions, lysozyme at mucosa
IgA production and *defensin* secretion at mucosal surface.
Microbiological
Competition for limited nutrients and adhesion sites on food particles, mucus and intestinal mucosa.
Cooperative interactions between microorganisms
Generic and species composition of microbiota
Inhibition of allochthounous species by fermentation products including HS+, SCFA, phenolic compounds, deconjugated bile salts etc. Bacterial secretion of antagonistic substances such as bacteriocins
Synergistic effects of bacterial antagonism and local immunity in the mucus layer and on the colonic mucosa

Functions of the microbiota

A common method to analyse the interaction between host and GI microbiota is the germ-free animal model which provides a "living test tube" (Berg, 1996). It is possible to keep experimental animals in germ-free conditions by delivering pups

through sterile caesarean section and rearing them aseptically. The animals are bred in an isolator which is ventilated with filtered sterile air. These gnotobiotc (germ-free) animals can be colonized with one or more bacteria species. In this way it is possible to study their interaction with the host in a simplified system (Berg, 1996). However, germ free animals colonized with certain microorganisms can never be considered "true conventional animals", as they lack the influence (immunological, physiological, etc.) of an indigenous GI. Nonetheless these gnotobiotc models offer a good comparison between conventional and germ free animals, revealing the effects asserted on its host (Berg, 1996). The data obtained from these studies suggest that the microbiota has an important and specific role in metabolic, trophic and protective functions.

Before describing these functions, it is necessary to give a definition of colonisation resistance. The community that the GI microbiota develops at each site will consist of microbes able to adhere to the existing substrates and utilise the available nutrients and will be in a state of dynamic equilibrium. Any exogenous microbe attempting to colonise such a site will, therefore, be faced with a very difficult task, and the microbiota of that site is said to exhibit "colonisation resistance" as a consequence of its members having occupied all of the available physical, physiological, and metabolic niches (Wilson, 2004). The term is also used to refer to the capacity of a microbiota to control the number of potentially pathogenic members that may be present in that community. Colonisation resistance by the indigenous microbiota of a site involves a number of mechanisms, including: occupation of adhesion sites, alteration of the physicochemical environment, production of antagonistic substances and utilisation of all available nutrients within a site.

Colonization is the first type of interaction between host and external partners. Intestinal mucosa is coated with a layer of protective mucus that is continuously produced by epithelial cells. In the intestines two types of mucus are present:

1) an insoluble gel strongly attached to cells;

2) a viscous layer soluble in water that covers the gel.

The constituents of mucus are mucins (i.e. glycoproteins) whose structure is formed by gel and by polysaccharide components containing five types of carbohydrates: galactose, fucose, N-acetylglucosamine, N-acetilgalactosamine and sialic acid. These carbohydrates are combined in order to form different structures showing a multiple repertoire of linking sites for bacteria. Moreover this structure plays an important role in the cell to cell recognition process.

Bacteria come into contact with intestine through adhesion sites that are surface structures called adhesins. The nature of the adhesion site is genetically controlled by the host. Thus the genome of the host determines bacterial adhesion via mucin interaction. Various experiments have shown that normal microbiota always remains on the surface of the mucus, at the entrance of villi but never inside crypts. Initially in newborns the repertoire of adhesion sites is genetically coded. During this time, structures undergo a partial or total deterioration since mucus glycoproteins are degraded by the resident microbiota (*Peptostreptococcus micros* and members of genera *Ruminococcus* and *Bifidobacteria* produce a variety of glycoside hydrolases) (Falk et al., 1998). The first bacteria to colonize the newborn's gastrointestinal tract depend on an innate repertoire which varies according to the the individual genome. The adhesion sites evolve enabling new species to bind. This is the reason why every person has a peculiar intestinal microbiota.

The mucosal barrier function depends on the integrity of the physical mucosa and on the reactivity of the defensive factors i.e. mucosal blood flow, mucosal secretions and epithelial cells functionality (tight junctions, cell turnover, recognition and process of foreign compounds). This "barrier effect" is only one of the numerous functions that the intestinal microbiota perform.

Other functions are those related to host nutrition, detoxification, host development.

Functions related to host nutrition

The microbiota of the colon makes a significant contribution to the nutritional requirements of its host, and this microbial community may be considered an important digestive "organ". In the colon bacteria have access to nutrients**,** i.e**.** all of the food chemicals eaten by the host not yet absorbed (such as fibres and indigestible sugars), materials derived from the host (mucus, death cells) and various metabolites resulting from bacterial enzyme activities. Quantitatively, the most important dietary constituent to escape digestion in the upper regions of the GI is starch: some experimental evidence has shown that in individuals with a typical Western diet, approximately 10% of dietary starch reaches the colon, but the proportion can be much higher in countries where starchy foods constitute a larger proportion of the diet.

The other main group of undigested polysaccharides reaching the colon are non-starch polysaccharides which constitute the "dietary fibre" (plant cell wall components, storage polymers and exudates).

A number of low-molecular mass carbohydrates also reach the colon, like lactose, galacto-oligosaccharides and fructo-oligosaccharides.

Finally, host macromolecules – such as mucins, glycosphingolipids, hyaluronic acid and chondroitin sulphate- are also important sources of carbohydrates for colonic microbes.

Of the five principal genera present in adult human colon (*Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus*, and *Fusobacterium*), only *Fusobacterium* contains species that usually don't utilize sugars (Falk et al., 1998). In addition, the ability to degrade complex carbohydrates is not common among all strains. Intracellular and secreted enzymes transform simple carbohydrates into pyruvic acid via fermentation processes. The metabolic endpoint is the generation of short-chain fatty acids (SCFA as butyric acid, acetic acid, propionic acid, lactic acid). Acetic acid is the dominant substrate and butyric acid is the main source of energy (assessed on

70%) for colonic cells and it is a decisive factor for cellular differentiation (it stabilizes mitosis, promotes cellular repair and increases DNA levels) (Hagiage, 1994). In particular SCFAs and lactic acid are absorbed and serve as energy substrates for colonocytes (butyric acid), liver cells (propionate and lactate), and peripheral tissues. In addition to short chain fatty acids, the other major waste product of glucose fermentation are gases, principally hydrogen, carbon dioxide, and methane. Volatile fatty acids are absorbed by intestinal cells and their concentration regulates the intestinal pH, which influences the activation of different bacterial enzymes. Their action is correlated with the modification of intraluminal contents that may reduce the production of carcinogenic metabolites (Hagiage, 1994).

In addition to carbohydrates, the colon also receives proteins and peptides from several sources (the diet, the exfoliated epithelial cells, and pancreatic enzymes); they are rapidly degraded by a variety of microbial proteases and peptidases. Many colonic microbes can ferment these aminoacids to generate a range of products, including SCFAs. These important acids are also used as a precursor for the synthesis of mucosal lipids.

A number of vitamins are present in the colon,too and derive both from the diet and from the colonic microbiota – particularly *Bifidobacterium* spp., *Clostridium* spp., amd enterobacteria. Vitamins produced by colonic bacteria include biotin, vitamin K, nicotinic acid, folate, riboflavin, pyridoxine, vitamin B_{12}, and thiamine.

In conclusion, the results of this complex nutrition activity of the host are: the recovery of metabolic energy and absorbable substrates for the host; the supply of energy and the production of nutrition necessary for the bacterial growth and proliferation.

Functions related to detoxification

It has been shown that the metabolic capabilities of the colonic microbiota can produce detoxification of potentially harmful dietary constituents. In fact, it has been

demonstrated that many microbial inhabitants of the human colon can affect the mutagenicity of the heterocyclic aromatic amines by binding to them and/or by somehow modifying their structure (Kassie et al., 2001). On the whole, Gram-positive species (e.g. *Lactobacillus* spp., *Clostridium* spp. and *Bifidobacterium* spp.) have a stronger effect in reducing the mutagenicity of these compounds. In humans the consumption of *L. casei* or *L. acidophilus* has proven successful in reducing greatly the urinary and fecal mutagenicity linked to the ingestion of meat.

Plant glycosides are non-toxic, low-molecular mass compounds widely distributed in fruits, vegetables, tea and wine. In the colon, the sugar moieties of these compounds are removed by β-glicosidases, yielding aglycones. Many of these compounds are toxic, mutagenic, or carcinogenic. However, the flavonoids liberated from flavonoid glycosides in the colon also have protective effects against other mutagens and carcinogens. Such compounds include quercetin, rutin, myricetin, and morin.

Functions related to host development

There are several examples in the animal and plant kingdoms of indigenous microbes affecting the development of host tissues. Most informations regarding the role of microbes in mammalian development has been obtained by comparative studies involving germ-free animals. The lack of an indigenous microbiota can produce serious consequences on the anatomy and the physiology of an animal.

One of the major roles the intestinal microbiota plays is in stimulating the growth and differentiation of epithelial cells. In addition, gut microbes also appear to have a role in the maturation of the gut that occurs during weaning in mice. During this period, the levels of ileal ephitelial lactase decrease, and this coincides with colonisation of the gut by *B. thetaiotaomicron*.

It is well established that the indigenous microbiota plays a key role in the development of a competent immune system. Since the gut-associated lymphoid tissue (GALT) contains the largest collection of immunocompetent cells in the human

body, most studies have included the GI tract and its microbiota. Several structural and functional abnormalities are present in the immune system of germ-free animals: including low density of lymphoid cells in the gut mucosa, low circulating concentrations of antibodies, small specialised follicle structures etc. However, the exposure of the gut mucosa to the indigenous microbiota has a dramatic effect on GALT. Consequently, the number of intra-epithelial lymphocytes expands greatly; germinal centres with antibody- producing cells appear in follicles and in the lamina propria; the levels of circulating antibodies increase; and increased quantities of IgA are secreted into the gut lumen.

As described before, a healthy intestinal epithelium, in association with an established and stable intestinal microbial population, represents a vital barrier against the invasion or the uptake of pathogenic microrganisms, antigens and harmful compounds from the gut lumen. Specific immune responses are evoked by the specialized antigen transport mechanisms in the villus ephitelium and Peyer's patches. In the past the positive function of gut microrganisms in human health wasn't properly considered and most attention was drawn to the enteric pathogens and factors leading to gastrointestinal disorders or "dysbiosis". Now it is generally believed that a stable barrier, typical of healty individuals, can ensure the protection of the host and serve as support for the normal intestinal function and immunological resistance. Researchers consider GALT as the largest "immune organ" in the human body, and this "barrier" serves for the intrinsic protection against infective agents. Around 80% (10^{10}) of all immunoglobulin-producing cells are found in the small bowel and the gut microbial population is essential for the mucosal immune stimulation and the amplification of immunocompetent cells. Numerous physiological functions have been ascribed to the "normal" gut microbial population; the following are some of the most relevant:

- Maintenance and restoration of barrier function
- Stimulation of the immune system
- Maintenance of mucosa nutrition and circulation

- Improvement of bioavailability of nutrients
- Stimulation of bowel motility and reduction of constipation

Inflammatory bowel diseases

Inflammatory bowel diseases (IBDs) are chronic, relapsing inflammatory disorders of the intestine. The ulcerative colitis affects the colon, while the Crohn's Disease most commonly affects the distal small intestine and proximal large intestine. Diarrhoea and abdominal pains are among the most frequent clinical features of IBD. It has been observed that while some IBD patients enjoy good health and long periods of remission, others suffer from chronic poor health and a significant disruption of lifestyle. The reasons for IBDs incidence are still to be fully understood, but current evidence suggests that IBD may result from the interaction between genetic factors and occurrence of intestinal inflammation early in life. The resulting dysregulation of the immune system produces the chronic, relapsing conditions. Observations from human cases and experimental animal studies suggest that the intestinal microflora, or their metabolic products, could play a fundamental role either as targets for immune cells, or facilitators or amplifiers of inflammations, although the specific aetiological agents are, as yet, unknown.

At present investigations about the use of probiotics and prebiotics in the treatment of IBDs have shown promising perspectives. As a matter of fact, the efficacy of probiotic feeding in animal models of IBD and other non-pathogenic strains of *E. coli* is comparable to the performance of antibiotics.

Chapter 2. Genus *Lactobacillus* and *Bifidobacterium*

Genus *Lactobacillus*

The genus *Lactobacillus* belongs to the lactic acid bacteria (LAB), a group of Gram-positive, catalase-negative bacterial species which are able to produce lactic acid as the main end-product of the carbohydrate fermentation.

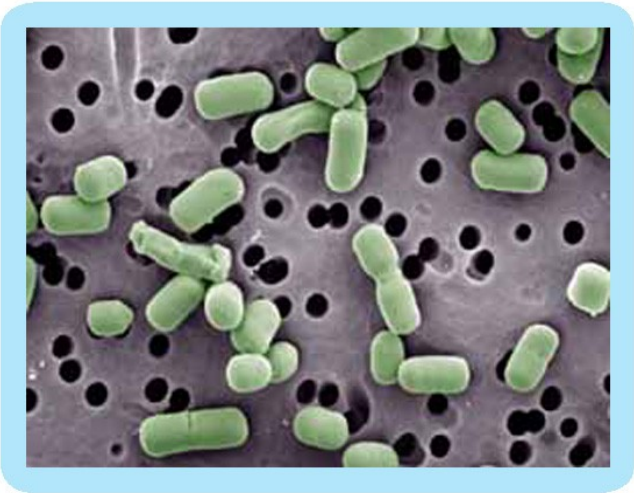


Fig.2. *Lactobacillus brevis*, image from SEM

Lactobacillus is a well-characterized genus belonging to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae*.

They are gram-positive non-sporeforming rods that are catalase negative, usually nonmotile, or motile by peritrichous flagella.

Their growth temperature ranges from 20 to 53°C, the optimum being between 30 and 40°C and their pH ranges from 5.5 to 6.2. They are aerotolerant anaerobes, and the microaerophilic atmosphere with 5 to 10% CO_2. enhance their growth. Glucose is used either homofermentatively or heterofermentatively, and they have complex

nutritional requirements for amino acids, carbohydrates, peptides, nucleic acid derivatives, vitamins, salts, fatty acids, or fatty acid esters.

Lactobacillus includes 113 recognized species and 16 subspecies and the type species is *Lactobacillus delbrueckii* Leichmann 1896 (Beijerinck, 1901). The genus *Lactobacillus* is very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. This heterogeneity can be seen in the range of moles percentage G+C of the genomic DNA of species included in the genus, which ranges range being 32 to 54%.

Metabolism and nutritional requirements

Lactobacilli possess efficient carbohydrate fermentation pathways coupled to substrate level phosphorylation. A second substrate level phosphorylation site is the conversion of carbamyl phosphate to CO_2 and NH_3.

Two main sugar fermentation pathways can be identified among lactobacilli: the Embden-Meyerhof pathway which results almost exclusively in lactic acid as an end product (homolactic fermentation) and the 6-phosphogluconate pathway producing significant amounts of other end products such as ethanol, CO_2, acetate, formate, or succinate, in addition to lactic acid (heterolactic fermentation). Each species has its own exact nutritional requirements which are often strain specific. In general, they require carbohydrates as energy and carbon sources as well as nucleotides, amino acids, and vitamins. Thiamine is necessary only for the growth of the heterofermentative lactobacilli, while pantothenic acid and nicotinic acid are required by all species. The requirements for riboflavin, pyridoxal phosphate, folic acid and p-aminobenzoic acid vary widely among the various species, riboflavin being the most frequently required, whereas biotin and vitamin B_{12} are requested by only a few strains.

Habitat of Lactobacilli

Lactobacilli are widely distributed in nature and have been isolated from various sources. They are almost ubiquitous: we can find Lactobacilli also in environments where carbohydrates are available, such as dairy products, fermented meat, sour doughs, vegetables, fruits and beverages.

They are part of the indigenous microbiota of the oral cavity, the gastrointestinal tract and the vagina in humans and animals, are isolated from plants, and are essential in the production of fermented foods such as dairy products, cured meats, marinated fish, wines, and silages (Morishita et al., 1981).

They constitute some of the most common gram-positive bacteria in the human microbiota. Lactobacilli are scattered throughout the entire gastrointestinal tract; from the upper part of small bowel, where they survive thanks to their acid resistance, to the large bowel (colon), where they reach a concentration of 10^6-10^8 bacteria per gram of intestinal contents.

In the oral cavity *L. casei*, *L. acidophilus*, and *L. fermentum* are present. In the gastrointestinal tract, lactobacilli can be found at different levels; qualitative and quantitative variations exist among individuals in relation to individual, ethnic, and nutritional factors (Barbés, 2001). The most frequent lactobacilli in the gastrointestinal tract belong to the following species: *L. acidophilus* and *L. fermentum* in the stomach; *L. acidophilus*, *L. fermentum*, and L. salivarius in the ileum; and *L. fermentum* and *L. salivarius* in the large intestine. Other strains have been isolated in smaller numbers: *L. casei*, *L. plantarum*, *L.brevis*, *L. buchneri*, *L. crispatus*, and *L. reuteri*.

Lactobacillus spp. include the dominant members of the vaginal microbiota, at 10^7 to 10^8 CFU/g of vaginal fluid in healthy premenopausal women, constituting 61.5 % of all microorganisms isolated; *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. Iners* are the species most frequently found (Jin et al., 2007).

Lactobacilli have been found also on the skin, in nasal and conjunctival secretions, in the ear, in breast milk, and in sperm. *Lactobacillus* spp. are present in the intestines of pigs, chicken, dog, mice, rats, and hamsters (Mitsuoka, 1992). In addiction, it is possible to find lactobacilli in sewage and in plant material**.**

Recently some species have been isolated from infections, above all there are several reports about *L. rhamnosus*.

Taxonomy, probiotic action and application

According to Euzéby (2007), the genus *Lactobacillus* is composed of 113 species. The reclassification of a number of strains and the descriptions of several new species have been published recently in the *International Journal of Systematic and Evolutionary Microbiology* (Euzéby, 2007).

Lactobacillus spp. are among the most frequent and better characterised microorganisms used as a probiotic. Important considerations in the choice of a probiotic include safety, functional aspects and technological aspects (Donohue et al. 1998). The genus Lactobacillus has a long history of safe use, and most strains are considered commensal microorganisms with no pathogenic potential.

Most probiotic formulation usually contains one or several selected strains of bacteria generally recognised as safe (GRAS). The *Lactobacillus* genus has received the recognition of 'Long-standing Presumption of Safety' status (Bernardeau et al., 2008).

Food and industrial applications

With regard to *Lactobacillus* spp., the following strains are used commercially: *L. acidophilus, L. plantarum, L. casei, L. fermentum, L. johnsonii, L. paracasei, L. reuteri, L. rhamnosus* and *L. salivarius* (Sanders and Veld, 1999; Senok et al., 2005). Each strain could be used more specifically for a particular illness or disorder, that is

why the future of probiotics will depend on the ability of choosing suitable strains for a given person, to treat specific disorders.

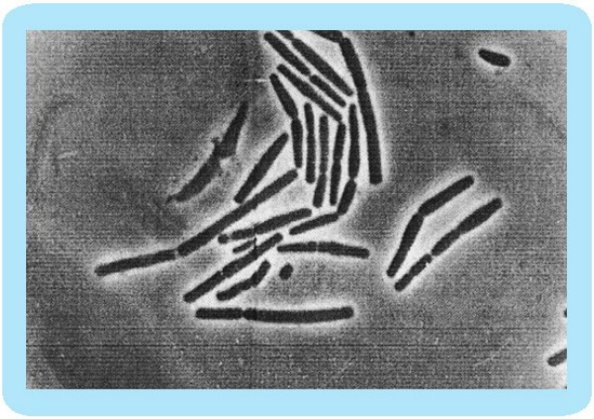


Fig. 3. *Lactobacillus acidophilus* isolated from food.

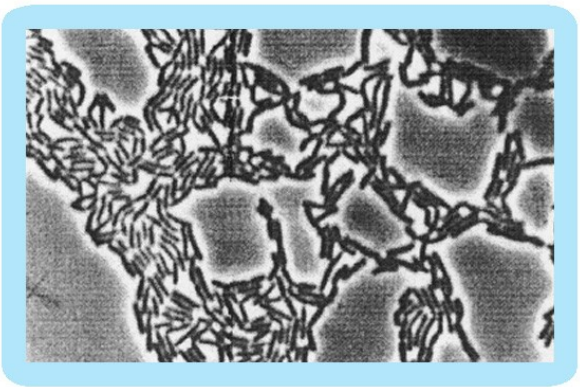


Fig. 4. *Lactobacillus casei*

Some commercially probiotic products containing lactobacilli. Some examples are indicated in table below:

Table 2. *Lactobacillus* species in intestines of humans and animals.

***Lactobacillus* species included in probiotic preparation**	**Type of product**
L. acidophilus NCFM	Fermented products, dietary supplement and toddler formula
L. acidophilus R0052	Pharmaceutical preparation for microbiota replacement
L. acidophilus LB	Pharmaceutical preparation for microbiota replacement
L. casei DN114001	Dairy fermentations
L. casei Shirota	Dairy fermentations
L. fermentum VRI003	Pharmaceutical preparation for microbiota replacement
L. johnsonii Lj-1 (La-1)	Pharmaceutical preparation for microbiota replacement
L. paracasei CRL 431	Dairy fermentations
L. paracasei F19	Pharmaceutical preparations for microbiota replacement
L. plantarum 299 V	Fruit juice, dairy fermentations, pharmaceutical preparation
L. reuteri RC 14	Pharmaceutical preparation for microbiota replacement
L. reuteri SD2112	Pharmaceutical preparation for microbiota replacement
L. rhamnosus GR-1	Pharmaceutical preparation for microbiota replacement
L. rhamnosus R0011	Pharmaceutical preparation for microbiota replacement
L. rhamnosus 271	Dairy fermentations
L. rhamnosus GG	Pharmaceutical preparation for microbiota replacement
L. rhamnosus LB21	Dairy fermentations and fruit juice
L. rhamnosus HN001 (DR 20)	Dairy fermentations and pharmaceutical preparation
L. salivarius UCC118	Dairy fermentations

Many Lactobacilli have been used in food fermentation processes, in functional foods as probiotic microorganisms and in pharmaceutical preparations for microbiota replacement.

An application of *Lactobacillus* spp. in industry is as a food preservative because bacteriocins, organic acids and other additional metabolites produced during the

fermentation of foods can inhibit growth of pathogenic organisms, thus extending the shelf life of fermented foods.

Lactobacillus spp. also produce heteroplysaccharides; microbial exopolysaccharides are biothickeners that can be added to a variety of food products, where they serve as viscosifying, stabilizing, emulsifying and gelling agents. Dextrans, a microbial exopolysaccharides can be used in the pharmaceutical and medical industries.

In addition, commercial strains can be genetically modified to improve inherent properties, to introduce desirable characteristics or to remove the unwanted ones.

Medical applications

Lactobacilli are useful in the treatment of gastrointestinal diseases (antibiotic associated diarrhea, traveler's and infectious diarrhea, ulcers related to *Helicobacter pylori*, lactose malabsorption symptoms, gastroenteritis and inflammatory bowel disease) and genitourinary infections. They have also other clinical applications (they reduce the recurrence of early atopic disease in curing or preventing atopic eczema in infants, they have moderate hypocholesterolemic properties and they cause a significant reduction in the severity of pneumonia in children).

Lactobacilli in animals

In contrast to humans, the proximal portions of the digestive tracts of pig, mouse and rat harbour large populations of bacteria (about 10^8 bacteria per gram of content). The stomach of pigs, mice and rats is lined partly with a non-glandular, squamous stratified epithelium, unlike the stomach of men, which is lined with a glandular mucosa. These regions of rats are colonized by lactobacilli adhering directly to the epithelium. Lactobacilli from this layer continuously inoculate the digesta, so they can be found in large numbers throughout the gastrointestinal tract.

The cell counts for lactobacilli in the rodent forestomach, colon and cecum can be higher than 10^8 bacteria per gram of content with slightly lower counts in the duodenum (10^6–10^7), jejunum and the ileum (10^7–10^8).

In ilea and large intestines of pigs *L. amylovorus*, *L. johnsonii* and *L. reuteri* are numerically dominant; a new species, *L. pantheris*, was detected in the feces of jaguars. *L. salivarius* is the predominant organism in the crop of birds.

In addition, *L. aviarius* with the subspecies *aviarius* and *araffinosus* were isolated from the feces and alimentary tract of chickens and ducks. Other adhering microorganisms were isolated from the nonsecreting area of the horse stomach: *L. salivarius*, *L. crispatus*, *L. reuteri* and *L. agilis*.

Lactobacillus equi was not recovered at that time but occurred in the feces of horses.

The *Lactobacillus* strains that adhere to epithelial cells show specificity for an animal host, even if some exceptions occur.

Relatively little is still known about the *Lactobacillus* population of the ruminants and especially of the rumen of adult animals. The numbers of lactobacilli vary according to the age and diet of the animal. In adult sheep and cattle, LAB constitute usually only a minor component of the microbial flora of the rumen.

Many (but not all) LAB are sensitive to animal feed antibiotics.

Although insects may play a role as vectors for the dissemination of lactobacilli, little is known about the lactobacilli in these animals.

The Bifidobacteria

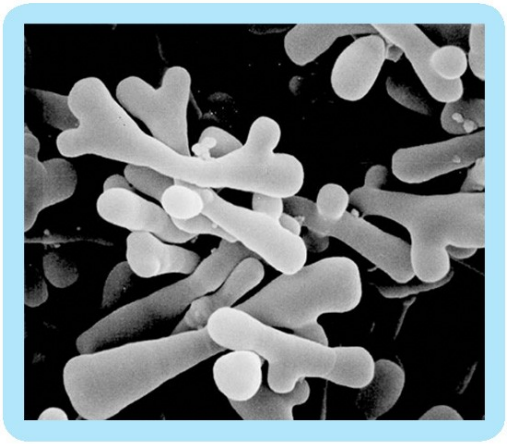


Fig. 5. Scanning electron micrographs of *Bifidobacterium* spp.

In 1900, Tissier observed and isolated in the feces of breast-fed infants a bacterium with a strange and characteristic Y shape and called him "Bacillus bifidus" (Tissier, 1899). This bacterium was anaerobic, Gram-positive and did not produce gas during its growth (Tissier, 1899). He proposed its inclusion in the family Lactobacillaceae. For a long time, bifidobacteria were included in the genus *Lactobacillus*. In the 8th edition of Bergey's Manual of Determinative Bacteriology bifidobacteria were classified for the first time in the genus *Bifidobacterium* and comprised eight species. Nowadays, according to *Taxonomic Outline of the Prokaryotes*, the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, sub-class *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. The other genera belonging to this family are: *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia* and *Scardovia*.

At present the species included in the genus Bifidobacterium are:

Bifidobacterium adolescentis, *Bifidobacterium angulatum*, *Bifidobacterium animalis (with two subspecies B. animalis subsp. animalis and B. animalis subsp. lactis)*, *Bifidobacterium asteroids*, *Bifidobacterium bifidum (type species)*, *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium*

choerinum, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium indicum*, *Bifidobacterium longum*, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum (with the two subspecies B. pseudolongum subsp. pseudolongum and B. pseudolongum subsp. globosum)*, *Bifidobacterium psychraerophilum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium thermacidophilum (with the two subspecies B. thermacidophilum subsp. thermacidophilum and B. thermacidophilum subsp. porcinum)*, and *Bifidobacterium thermophilum*.

Bifidobacterium longum is the most common species in the human gut and has been isolated both in infants and adults (Biavati et al., 2000). Scardovi et al. (1979) outlined the strong genetic link between *B. longum* and *B. infantis* with DNA-DNA homologies values in the 65%-80% range. Moreover a group of strains isolated in calves with a 75%-80% homology, with both *B. longum* and *B. infantis*, was described. Researchers concluded that *B. infantis* and B. *longum* can form a single species, a "continuum", the middle position of which is taken by the strains isolated in calves. Recently (Mattarelli et al. 2008), with the aid of different genotypic techniques mattarelli and collaborators proposed a new classification of 3 biotypes of *B. longum* in 3 subspecies: *B. longum* subsp. *longum* subsp. nov., *B. longum* subsp. *infantis* comb. nov. and *B. longum* subsp. *suis* comb. nov.

Physiology and metabolism

Bifidobacteria are Gram-positive polymorphic branched rods that occur singly, in chains or in clumps. They are non-spore forming, non-motile and non-filamentous. They are anaerobic : their sensitivity to oxygen changes in relation to the species and the different strains of each species. Bifidobacteria are chemoorganotrophs, having a

fermentative type of metabolism. They produce acid but not gas from a variety of carbohydrates. They are catalase negative (with some exceptions). Their genome GC content varies from 42 mol% to 62 mol% (Biavati and Mattarelli, 2001).

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

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

Bifidobacterium produces lactic and acetic acid from glucose.

The global equation is:

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

This peculiar metabolic pathway is called “fructose-6-phosphate shunt” or “bifidus shunt”. The key enzyme of this pathway is fructose-6-phosphate-phosphoketolase, which was considered a taxonomic character for the identification on the genus level (Biavati and Mattarelli, 2001). Different species produce variable amounts of acetate, lactate ethanol and formate under the same conditions. Bifidobacteria utilize a great variety of mono- and disaccharides as carbon sources and are able to metabolize also complex carbohydrates that are normally not digested in the small intestine. This feature should give an ecological advantage to colonizers of the intestinal environment where complex carbohydrates, such as mucin, are present either because they are produced by the epithelium of the host or because they are introduced through diet.

Habitat

The presence of bifidobacteria in the alimentary tracts of human adults and infants has stimulated much interest among bacteriologists and nutritionists. The number and

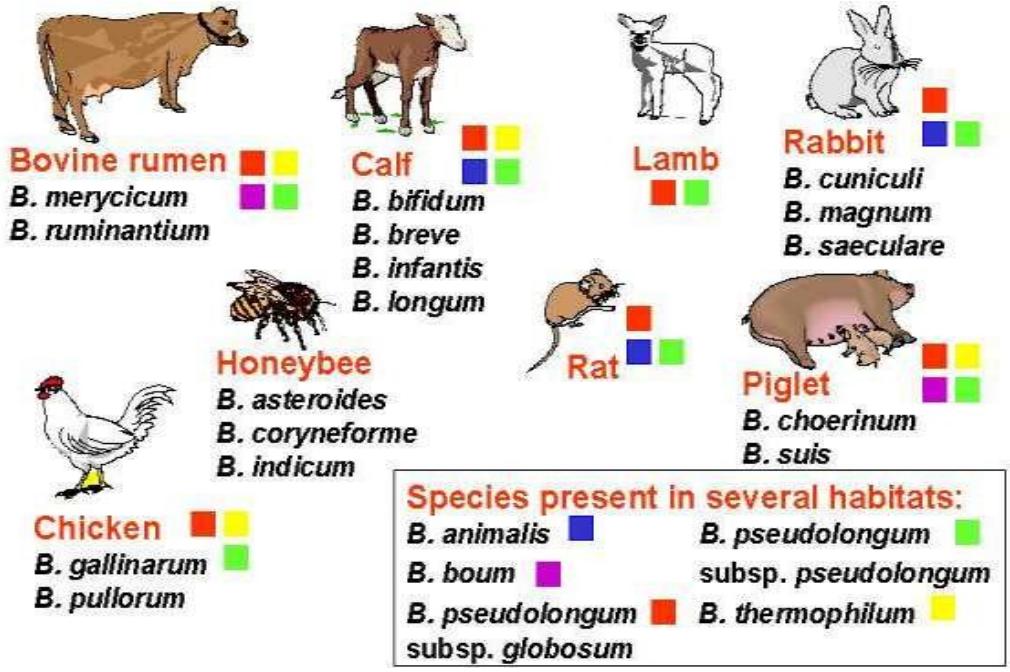
the composition of the microbial populations in different regions of the GI tract are controlled by many factors. In the intestinal tract of animals and humans, bifidobacteria coexist with a large variety of bacteria, most of which are obligate anaerobes; the components of this microflora are different in the different areas of the tract.

Studies on the ecology of bifidobacteria performed at the Institute of Agricultural Microbiology at Bologna University, Italy, have led to the isolation of a large number of strains (at present more than 7,000) from many different habitats.

Studies on the distribution of bifidobacteria have pointed out that the most represented species in the faeces of newborns are: *B. breve* and *B. infantis*, whereas in the faeces of adults *B. adolescentis*, *B. angulatum*, *B. dentium* and *B. gallicum* are prevalent; *Bifidobacterium* spp. are found in both newborns and adults, like *B. bifidum*, *B.longum* (the two most representative), *B. catenulatum* and *B. pseudocatenulatum*. *B. breve*, *B. dentium* and *B. longum* are normally colonizers of human vagina. In "pathological conditions" such as in dental caries it is possible to find *B. denticolens*, *B. dentium* and *B. inopinatum*, and these same species, with *B.infantis* and *B. longum*, are also present in case of hypochlorhydria.

Furthermore, bifidobacteria have been isolated from many animals, and among the insects, from honeybees. In general, *Bifidobacterium* species are specific either for humans or for animals; with the only exception of the same *Bifidobacterium* species found in the intestinal microflora of suckling calves and breast-fed infants**.** Some species, 12 out of 16, are host-specific, and they are typical of a given animal habitat (Fig. 6).

Fig. 6. *Bifidobacterium* species found in the animals.



Eleven species of *Bifidobacterium* have been found in sewage: *B. adolescentis*, *B. angulatum*, *B.breve*, *B. longum*, *B. pseudocatenulatum* are from humans, *B. animalis*, *B. choerinum*, *B. pseudolongum* subsp. *globosum*, thermophilum from animals and *B. minimum* and *B. subtile* are found only in sewage.

Bifidobacteria in animals

Studies on the intestinal microflora, carried out mostly on domestic animals, revealed a complex microflora: *Bacteroides*, eubacteria, anaerobic lactobacilli, anaerobic Gram-positive cocci, spirillaceae and often bifidobacteria. Almost all chickens, dogs, pigs, rats and hamsters presented bifidobacteria, although in a smaller quantity than lactobacilli. Mice, rabbits and horses rarely displayed bifidobacteria, and cats and minks never had them. Many factors influence the composition of bifidobacteria microflora in animals: the age, the species and the diet of the host.

Some species apparently are host-specific: *B. magnum* and *B. cuniculi* have only been found in rabbit faecal samples, *B. pullorum* and *B. gallinarum* only in the intestine of chickens and *B. suis* only in piglet faeces (Matteuzzi et al., 1971; Fig. 6).

Chapter 3. Probiotics

History of Probiotics

The term *probiotic*, meaning “for life,” is derived from the Greek language and it is currently used to name bacteria associated with beneficial effects for humans and animals. The original observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, the Russian Nobel Prize working at the Pasteur Institute at the beginning of the last century; Metschnikoff (1908) in his bestseller *The Prolongation of Life* was probably the first to advocate, or rather postulate, the health benefits of LAB associated with fermented milk products. He hinted that the longevity of the Caucasians could be related to the high intake of fermented milk products and that the intake of yogurt containing lactobacilli might result in a reduction of toxin-producing bacteria in the gut and that this could increase the longevity of the host. Tissier, a French paediatrician, recommended the administration of bifidobacteria to infants suffering from diarrhea, claiming that bifidobacteria supersede the putrefactive bacteria that cause the disease (Doderlein, 1892). The expression “probiotic” was probably first defined by Kollath in 1953 (Kollath, 1953), when he proposed the term to identify all organic and inorganic food complexes as “probiotics,” in contrast to harmful antibiotics in order to upgrade such food complexes as supplements. In his publication “Anti- und Probiotika," Vergio (1954) compared the detrimental effects of antibiotics and other antimicrobial substances with favorable factors (“Probiotika”) on the gut microbiology.

The word "probiotic" was coined in 1965 by Lilly and Stillwell (1965) to describe “substances secreted by one microorganism which stimulates the growth of another” and thus was contrasted with the term *antibiotic*. It may be because of this positive and general claim of the definition that the term *probiotic* was subsequently applied

to other substances and gained a more general meaning. In 1971 Sperti (Sperti, 1971) applied the term to tissue extracts that stimulate microbial growth. Parker (1974) was the first to use the term *probiotic* in the sense that it is used today. He defined probiotics as "organisms and substances which contribute to intestinal microbial balance." The use of the word *substances* in Parker's definition of probiotics resulted in a wide connotation that included antibiotics. Although numerous definitions have been proposed since then, none has proved completely satisfactory because of the need for additional explanations, e.g., with regard to statements such as "beneficial balance," "normal population," or "stabilization of the gut flora." In 1989 Fuller (Fuller, 1989) attempted to improve Parker's definition of probiotic with the following distinction: "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." This revised definition emphasized the requirement of viability for probiotics and introduced the feature of a beneficial effect on the host, which was, according to his definition, an animal. A similar definition was proposed by Havenaar and Huis in 't Veld (1992) "…mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora." Probiotics are best known by the average consumer in relation to food where they are defined by the EU Expert Group on Functional Foods in Europe (FUFOSE) as "viable preparations in foods or dietary supplements to improve the health of humans and animals". Salminen (1996) and Schaafsma (1996) broadened the definition of probiotics even further by no longer limiting the proposed health effects to influences on the indigenous microflora. According to Salminen, a probiotic is "a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host." According to Schaafsma, "Oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition." , In 2001, Schrezenmeir and Michael de Vrese proposed the following definition: "A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or

colonization) in a compartment of the host and by that exert beneficial health effects in this host". In 2002, FAO/WHO has adopted the definition of probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/ WHO, 2002).

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

The original assumption of Metchnikoff was that the dietary manipulation of gut microflora performed in order to increase the relative numbers of "beneficial bacteria" could contribute to the well being of the host. However he also stated that systematic investigations should be made on the relation of gut microbes to precocious old age, and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body."

It is necessary to assess the efficacy and safety of probiotics and this constitutes an important part of their characterization for human use. The probiotic potential of different bacterial strains, even within the same species, differs; different strains of the same species are always unique, and may have differing areas of adherence (site-specific), specific immunological effects, and actions on a healthy versus an inflamed mucosal milieu may be distinct from each other. Present studies are being carried out in order to characterize the healthy normal gut microbiota in each individual, analysing the species composition and the concentrations of different bacteria in each part of the intestine. They are aimed at fully understanding the interactions host-microbe within the gut, the microbe–microbe interactions within the microbiota and the combined health effects of these interactions in order to define the microbiota both as a tool for nutritional management of specific gut-related diseases and as a source of new microbes for future probiotic bacteriotherapy applications.

Microbes from many different genera are being used as probiotics (Table 3). The most commonly used strains are members of the heterogeneous group of lactic acid bacteria; lactobacilli, enterococci and bifidobacteria.

Table 3. Microrganisms used as probiotics.

Species and example strains	Health benefit and references
L. acidophilus La 5	Reduced antibiotic-associated diarrhea (Black et al. 1991)
L. casei Shirota	Shortening of rotavirus diarrhoea, reduced recurrence of superficial bladder cancer, immune modulation (Nagao et al., 2000).
L. johnsonii La1	Improved oral vaccination reduced colonisation by *H.pylori* (Felley et al. 2001)
L. *plantarum* 299v	Relief of irritable bowel syndrome , reduction of LDL-cholesterol (Niedzielin et al., 2001)
L. reuteri SD2112	Shortening of rotavirus diarrhoea (Shornikova et al., 1997)
L. rhamnosus GG	Shortening of rotavirus diarrhoea, immune modulation, relief of inflammatory bowel disease, treatment and prevention of allergy (Kalliomäki et al. 2001)
Bifidobacterium lactis Bb12	Treatment of allergy, shortening of rotavirus diarrhoea, reduced incidence of travellers diarrhoea Improved oral vaccination (Isolauri et al., 2001)

Effects of probiotics

The mechanism of probiotic action is still unknown but different approaches could be developed. According to Fuller (1989) and Huis in't Veld and Havenaar (1993) probiotic effect of lactic acid bacteria and bifidobacteria may be expressed by three main mechanisms of action:

1. Suppression of pathogenic microorganisms in the intestinal tract by:
a) production of antibacterial substances including primary metabolites, such as lactic acid, acetic acid, carbon dioxide, diacetyl, acetaldehyde, hydrogen peroxide and

bacteriocins; they are proteinaceous compounds with antimicrobial activities against other closely related bacteria;

b) competition for nutrients. In a paper about the large intestine Freter *et al.* (1983) claimed that the competition for limited nutrients (specific carbohydrates) is the determining factor withthe greatest scientific support;

c) competition for adhesion receptors on the gut epithelium. Probiotic strains can adhere specifically or non-specifically. Specific adhesion takes place when an adhesion on the bacterial cell binds to a receptor on the epithelial cell; this is commonly defined as a lock and key function. Non-specific adhesion is a more general phenomenon mediated by hydrophobic or electrostatic interaction. Although it may not have any partcular relevance in the colonisation of epithelia *in vivo,* it may possibly be important in the colonisation of luminal contents. For example, non-specific adhesion may enhance the substrate uptake and consequently the growth (Jonsson, 1992).

2. Alteration of microbial metabolism in intestinal tract:

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

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

3. Stimulation of immunity:

Recent reports have shown that orally administered lactobacilli can improve immune status by increasing the circulating and local antibody levels, the gamma interferon concentration, the macrophage activity and the number of natural killer cells. The inclusion of lactic acid bacteria as members of physiological indigenous microflora into the mucosa and the subsequent translocation to other organs is currently regarded as a crucial step for the development of the normal mucosal and systemic immunity.

Mechanism of competitive exclusion

The resident microflora exists in a symbiotic relationship with the host and receives a rich and continuous nutrient supply. It improves nutrient bioavailability to the host and augments disease resistance mechanisms of the host. The ability of lactic acid bacteria to reduce the gastrointestinal invasion of pathogenic bacteria has been described in several works (Bernet et al., 1994; Bernet et al., 1993). The protection afforded by the indigenous flora is thought to be related also by competitive exclusion and interference with the attachment to mucosal surface. Many indigenous and pathogenic bacteria specifically adhere to complex oligosaccharides associated with proteins and/or lipids of intestinal membranes. The inter- and intra-species diversity of intestinal glycosylation patterns/carbohydrate epitopes is well established. Furthermore, it is possible that the ability of lactic acid bacteria to compete with pathogens for adhesion to the intestinal wall is influenced by their membrane fluidity. This possibility was suggested by studies which claimed that the type and quantities of polyunsaturated fatty acids in the extracellular milieu influence the adhesive properties of lactic acid bacteria to the epithelium (Kankaanpaa et al., 2004).

The interaction of bacteria with binding sites may evolve direct recognition or recognition of cryptic receptors following the action of secreted exoglycosidase enzymes. A *Lactobacillus* strain was shown to competitively inhibit the adhesion of enteropathogenic *E. coli* to pig ileum and interfered with bacterial attachment to the mucosal layer of ileal conducts (Loones, 1989). Although *L. acidophilus* inhibits the adhesion of several enteric pathogens to human intestinal cells in culture, when pathogen attachment preceeded the treatment with *L. acidophilus*, no inhibitory interference occurred indicating that steric hindrance of site occupation is important in the inhibition of adhesion. Thus, the therapeutic use is limited to the preventive application and not to a curative goal once binding of the pathogen has occurred. In addition, a dose-dependent inhibition against cell adhesion of several pathogens has

been demonstrated only for one strain of *L. acidophilus* (LA1) (Beshkova et al., 1998).

A number of health effects are associated with the use of probiotics. These include:

Nutrient synthesis and bioavailability

It has been shown that the action of micro-organisms during the preparation of cultured foods or in the digestive tract can improve the quantity, availability and digestibility of some dietary nutrients. The fermentation of food with lactic acid bacteria increases the folic acid presence in yogurt, kefir and bifidus milk (Alm 1982). Similarly, niacin and riboflavin levels in yogurt rise with fermentation (Alm 1982). The lactic acid bacteria are known to release various enzymes and vitamins into the intestinal lumen. This has synergistic effects on the digestion, alleviating symptoms of intestinal malabsorption, and producing lactic acid, which lowers the pH of the intestinal content and prevents the development of invasive pathogens such as *Salmonella* spp. or strains of *E. coli* (Mallett et al. 1989; Mack et al. 1999). The bacterial enzymatic hydrolysis may enhance the bioavailability of protein and fat (Fernandes et al. 1987) and increase the production of free amino acids and short chain fatty acids (SCFA)**.** When absorbed these SCFAs contribute to the available energy pool of the host (Rombeau et al. 1990; Rolfe 2000) and may protect against pathological changes in the colonic mucosa (Leavitt et al. 1978; Leopold and Eileler 2000). SCFA concentration helps to maintain an appropriate pH in the colonic lumen, which is critical in the expression of many bacterial enzymes and in unfamiliar compounds and carcinogen metabolism in the gut (Mallett et al. 1989).

In addition to the nutrient synthesis, the action of microorganisms either during the preparation of cultured foods or in the digestive tract can, to a limited extent, improve the digestibility of some dietary nutrients. Several lines of evidence show that the appropriate strain of lactic acid bacteria (i.e. *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and other lactobacilli used in fermented milk products) in adequate amounts, can alleviate symptoms of lactose intolerance by delivering

enough bacterial lactase to the intestine and stomach where lactose is degraded to prevent symptoms in lactase non persistent individuls. (Kilara and Shahani 1975; Martini et al. 1991). Figure 7 is the representation of various functions and health benefits of probiotics.

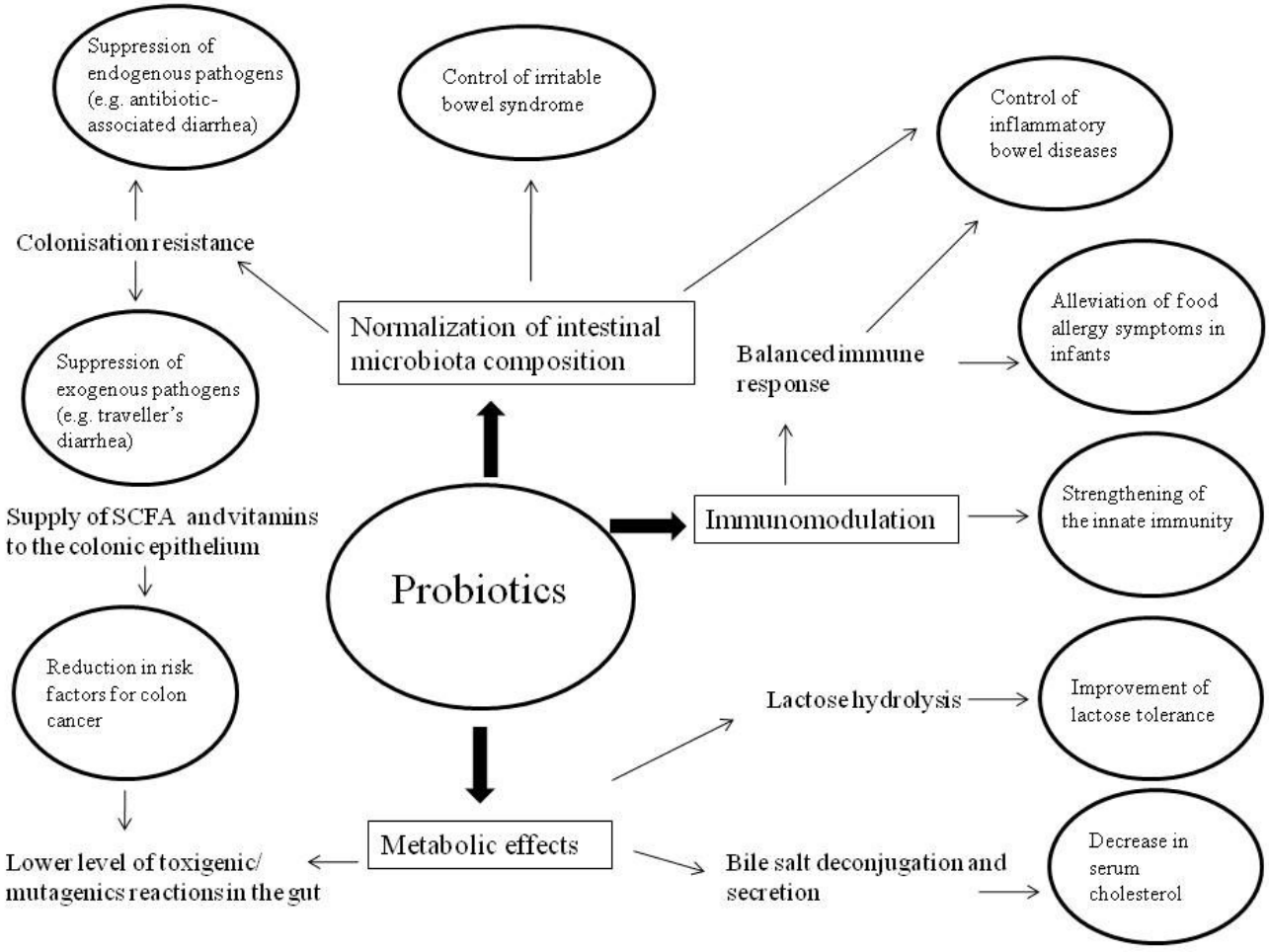


Fig 7. Various health benefits from probiotics consumption

Reduction of lactose intolerance

There is good scientific evidence about the alleviation of lactose intolerance symptoms by a specific probiotic lactic acid bacteria: several studies have reported that lactose-intolerant individuals suffers fewer symptoms if milk in the diet is replaced with fermented dairy products. The bacterial enzyme, B-galactosidase,

which can be detected in duodenum and terminal ileum after consumption of viable yogurt, is the factor that improves digestibility by the hydrolysis of lactose.

Diarrhoea treatment

Numerous studies have been carried out in this field and *Lactobacillus rhamnosus* GG has proved to be effective in the treatment of rotavirus diarrhoea, since it reduces the duration of diarrhoea to about half in children with rotavirus diarrhoea. When different lactic acid bacteria were compared for their effects on the immune response to rotavirus in children with acute rotavirus gastroenteritis differences between strains were observed. *Lactobacillus* GG was found to be more effective than other preparations (*Streptococcus thermophilus* and a *L. bulgaricus*, or a *L. rhamnosus* strain or a preparation containing *L. rhamnosus*). During convalescence the treatment with Lactobacillus GG was associated with an enhancement of IgA sASC to rotavirus and serum IgA antibody level. It was therefore believed that certain strains of lactic acid bacteria could promote systemic and local immune response to rotavirus. This may be of importance for protective immunity against reinfections.

Immune modulation by probiotics

As already evidenced in the previous chapter, the gut microflora is an important constituent in the intestines defence barrier. The gut microflora affects the development of gut-associated lymphoid tissue at an early age and consequently directs the regulation of systemic and local immune responsiveness, including hyporesponsiveness to antigens from micro-organisms and food. The success of a probiotic therapy can be seen in the normalisation of the increased intestinal permeability and the altered gut microecology, the improvement of the intestine immunological barrier functions and the alleviation of the intestinal inflammatory response. The targets for probiotic therapy are identified as clinical conditions involving impaired mucosal barrier function, particularly infectious and inflammatory diseases (Isolauri 2001).

Food allergy reduction

It is widely recognized that human beings are exposed to numerous environmental antigens through food. The intestinal mucosa can efficiently assimilate antigens encountered by the enteric route, but a high-level antigen exposure during the first few months of life may predispose individuals to allergic sensitization. Intestinal inflammation seems to be a predisposing factor in the increased sensitization of a subject. In the absence of intestinal microflora, antigen transport is increased. Studies have shown that intact cow's milk proteins can stimulate peripheral blood mononuclear cells to release pro-inflammatory cytokines in patients with cow's milk allergy (Heyman et al., 1995). However, it has been shown that the cow's milk proteins degraded by Lactobacilli, but not by trypsin or pepsin, may generate tolerogenic peptides from the native protein. These findings tend to substantiate the hypothesis that specific strains of intestinal microflora may contribute to the protection of the host against allergic sensitization (Hatakka et al., 2001). The management of food allergy is at present aiming at completely avoiding foods proven to cause symptoms as in the treatrment of infants with cow's milk allergy in which extensively hydrolyzed formulas are used to eliminate cow's milk antigens from the diet.

Inflammatory diseases and irritable bowel syndromes

Pouchitis and the Crohn's disease, as well as the irritable bowel syndrome (IBS) may be caused or aggravated by alterations in the gut flora including infection (Shanahan, 2000). Some studies support the potential role of probiotics in the therapy and prophylaxis and illustrate that combinations of strains may have a role in remediation (Gupta et al., 2000). The intestinal microflora is likely to play a critical role in the inflammatory conditions in the gut, and probiotics could remediate such conditions through modulation of the microflora.

Indeed selected probiotics have been observed to reduce the number of relapses and to prolong the period of remission. Interestingly, not only the lactic acid bacteria, *L.*

salivarius UCC118 and *L. rhamnosus* GG, but also *S. cerevisiae* (*boulardii*) and a strain of *E. coli* (Nissle) have proved effective in alleviating the symptoms of IBD (Gupta et al. 2000; Hamilton-Miller 2001). Clinical trials showed that in the IBS, *L. plantarum* 299v and DSM 9843 strains reduced abdominal pain, bloating, flatulence, and constipation (Steidler et al. 2000; MacFarlane and Cummings 2002).

Colorectal cancer

There is a diverse aetiology of colorectal cancer in which diet is clearly involved (Greenwald et al. 2001). Diets which are high in meat and fat or low in fibre can cause changes in the composition of the intestinal microflora, with increasing levels of *Bacteroides* and *Clostridium* and decreased levels of *Bifidobacterium* (Benno et al. 1991). This modification in the microflora composition is associated with an increase in faecal enzyme activity, *β*-glucuronidase, azoreductase, urease, nitroreductase and glycocholic acid reductase. These enzymes turn procarcinogens into carcinogens and may thus increase the risk for colorectal cancer. It has been observed that the consumption of selected lactobacilli reduces this faecal enzyme activity. Although it is still to be proven whether this also reduces the actual risk for colorectal cancer, most, but not all, epidemiological studies suggest that the regular consumption of fermented dairy products is related to a lower risk for certain types of cancer (Hirayama & Rafter 2000). Some positive effects of the probiotic lactic acid bacteria on the risk for colorectal cancer can therefore be anticipated although definite proof is still to be obtained.

Activity against *Helicobacter pylori*

Specific strains of lactic acid bacteria have been reported to inhibit many intestinal pathogens including *Helicobacter pylori*. The lactic acid bacteria are often able to survive the acidic gastric conditions and therefore it has been proposed that they may have a beneficial influence during the eradication of *H. pylori*, which is involved in the development of gastric ulcer. It has been reported that both the inhibitory

substances and the specific strains may influence the survival of *H. pylori* and studies have been conducted especially with a *L. johnsonii* strain. It has been shown that there is a good in vitro inhibition and that fermented milk containing the strain hase a positive effect when consumed during *Helicobacter* eradication therapy (Mitchetti et al., 1999).

Hepatic disease

The hepatic encephalopathy (HE) is a liver disease and its effects can be life threatening. The exact pathogenesis of HE still remains unknown. The probiotics *S. thermophilus*, Bifidobacteria, *L. acidophilus*, *L. plantarum*, *L. casei*, *L. delbrueckii bulgaricus*, and *E. faecium*, which show a definite therapeutic effect, have multiple mechanisms of action that could disrupt the pathogenesis of HE and lower portal pressure with a reduction in the risk of bleeding, which may make them preferable to conventional treatment (Cunningham-Rundles et al. 2000; De Santis et al. 2000; Gorbach 2000; Guslandi et al. 2000; Shanahan 2001; Solga 2003).

Hypertension

About 50-60 million people in United States are estimated to have hypertension, or elevated blood pressure. Antihypertensive effects have been documented in animal models and in mildly hypertensive adults for three compounds derived from the growth of certain lactobacilli: i) fermented milk containing two tripeptides derived from the proteolytic action of *L. helveticus* on casein in milk; ii) bacterial cell wall components from cell extracts of lactobacilli; and iii) fermented milk containing fermentation-derived gamma amino butyric acid. Systolic blood pressure decreased by 10-20 mm Hg. These results suggest that consumption of certain lactobacilli, or products derived from them, may reduce blood pressure in mildly hypertensive people. The viability of *Lactobacillus* is not required for the effect. Several fermentation-derived, but nonprobiotic, products have been developed.

Vaginosis

The vagina and its microbiota form a finely balanced ecosystem. Disruption of this ecosystem can lead to a microbiological imbalance and symptoms of vaginosis. Vaginosis used to be considered a mere annoyance, but it is now being studied for a role in serious conditions including pelvic inflammatory disease, pregnancy-related complications (low birth weight babies, etc.), and increased susceptibility to AIDS infection. Vaginosis can be caused by several different organisms, and in many cases, the causative agent may not be identified. What is known is that lactobacilli predominate in the healthy vagina, and a lack of lactobacilli (especially those producing hydrogen peroxide) is a risk factor for vaginosis. The lactobacilli are thought to maintain a favorable vaginal pH in the acidic range and to inhibit pathogens, possibly through the production of hydrogen peroxide and other antimicrobial factors. Intravaginal applications of lactobacilli have been somewhat effective in treating bacterial vaginosis.

Conditions of the genitourinary tract

The colon might thus be considered both a source of beneficial and of harmful bacteria for the urinary and genital tracts. It has been shown that both oral probiotics and vaginal suppositories of probiotics can reduce the incidence of recurrent urinary tract infection (McLean and Rosenstein 2000). One study suggests the possibility of vaginal contamination with faecal flora as the possible rationale for the effectiveness of this therapy (Cadieux et al. 2002).

Elevated blood cholesterol

Cholesterol is essential for many functions in the human body. It acts as a precursor to certain hormones and vitamins and it is a component of cell membranes and nerve cells. However, elevated levels of total blood cholesterol or other blood lipids are considered risk factors for developing coronary heart disease. Although humans synthesize cholesterol to maintain minimum levels for biological functioning, diet is also known to play a role in serum cholesterol levels. The extent of influence varies

significantly from person to person. Probiotic cultures have been evaluated for their effect on serum cholesterol levels. Clinical studies on the effect of lowering cholesterol or low-density lipid levels in humans have been inconclusive. Some studies on humans suggest that elevated blood cholesterol levels can be reduced consuming probiotic-containing dairy foods, but the evidence is not overwhelming.

Benefits for healthy subjects

Determining the potential health effects of probiotics for healthy subjects is difficult although this is of major importance since probiotics are mainly marketed for healthy subjects. The effects of probiotics on healthy subjects are likely to be limited only to the reduction of possible risks. The consumption of fermented dairy products maybe linked to a reduced risk for colorectal cancer. However, the evidence for this is rather circumstantial. Long term consumption of probiotics in non-fermented milk may reduce the risk for infections, the absence from day care due to illness and the use of antibiotics, at least for children (Hatakka et al. 2001). This study indeed suggests that probiotics can also be of benefit to the healthy consumer. Probiotics are often marketed as 'boosting the immune system'. For healthy individuals this may not be the case, since the immune system is likely to be working optimally (Spanhaak et al. 1998). However, in combination with oral vaccination, improved antibody titres have been observed with probiotics (Link-Amster et al. 1994).

Prebiotics and Synbiotics

The term *prebiotic* was introduced by Gibson and Roberfroid (1995) who exchanged "pro" for "pre," which means "before" or "for." They defined prebiotics as "a non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon." The major prebiotics are resistant dietary carbohydrates, but noncarbohydrates are not excluded from this definition. Prebiotics are believed to

stimulate selectively bacterial groups such as bifidobacteria, lactobacilli, and eubacteria resident in the colon which are considered particularly beneficial for the human host. Resistant short-chain carbohydrates (SCCs) are also called nondigestible oligosaccharides or low-digestible carbohydrates (LDCs). These SCC or LDCs offer interesting possibilities for inclusion into conventional food products for their "bifidogenic" effects. Several of such "candidate prebiotics" are currently under consideration by the industry for human consumption. Inulin and fructo-oligosaccharides (FOSs) are considered as typical "bifidogenic factors" and are probably the most commonly used prebiotics on the market.

Other promising prebiotic oligosaccharides under consideration are galacto-oligosaccharides, isomalto-oligosaccharides, soybean oligosaccharides, lactosucrose, and xylooligosaccharides.

It is generally agreed that there are a number of beneficial effects of prebiotics, particularly related to the favorable influence on the small bowel by improved sugar digestion and absorption, glucose and lipid metabolism, and protection against known risk factors of cardiovascular disease. In the colon, the fermentative production of SCFAs is to be considered a fundamental beneficial feature linked to the primary prevention of colorectal cancer. Other confirmed effects from prebiotics are related to the low energy value (<9 kJ/g) resulting from their nondigestibility, to an increase in stool volume, to the modulation of the colonic flora by stimulation of beneficial bacteria and the inhibition of "undesirable" bacteria. Some of these effects , not confirmed yet, concern the prevention of colorectal cancer, the modulation of the immune response, the prevention of intestinal infections and the reduction of the serum cholesterol levelas well as an improved bioavailability.

By definition, the word synbiotic refers to a product in which a probiotic and a prebiotic are combined. The growth of a probiotic strain that is able to utilize a prebiotic will be selectively stimulated in the gut. This combination of pre and probiotics in a single product has been shown to offer more benefits than either substance on its own.

Chapter 4. Applications of probiotics

Industry stakeholders for probiotics include businesses involved in conventional and speciality foods and beverage, dietary supplements, consumers' health care, biopharmaceuticals, veterinary health care, and agriculture.

Probiotics in food industry

Functional foods as a marketing term appeared in Japan in the late 1980s and it was used to describe foods fortified with ingredients capable of producing health benefits. Nowadays, the consumer pays a lot of attention to the relation between food and health. As a consequence, the market for functional foods has shown a remarkable growth over the past few years. Probiotic products represent a remarkable growth area within the functional food group and intense research efforts are under way to develop dairy products into which probiotic organisms are incorporated. The consumer market for probiotic food is about 1.4 billion in Western Europe (Saxelin, 2008). The biggest sector is represented by yogurts and desserts, with sales of about 1 billion euros, and the rest of the market is primarily about probiotic milks. The food supplement business is estimated at 10% of the total market for probiotics. Probiotics are added to both fresh and fermented dairy products (i.e. milk, fermented milk, and yogurt). The most popular format of probiotic, however, is the "daily-dose" drink, which was introduced to Europe in 1994 by the Japanese company Yakult. The differences between the US and European markets for probiotics may be easily explained by the annual per-capita consumption of fermented milk products (including yogurt) in Europe (35-45 L/person/year), which is considerably higher than in North America (4-5 L/person/year) (Saxelin, 2008).

In probiotic foods, probiotics can be added simultaneously with the standard cultures in the fermentation tank, or combined to form the final product, after fermentation has taken place.

The use of food additives is regarded as unnatural and unsafe. Yet, additives are needed to preserve food products from spoilage and to improve the organoleptic properties. The demand for a reduced use of additives and processing has put the food industry under pressure to look for alternatives. In food fermentation, one of the key points for intervention seems to be at the level of the starter culture. Probiotics, that are able to produce antimicrobial substances, represent a way of replacing chemical additives by natural compounds, proving at the same time the consumer with new, attractive food products.

A starter culture can be defined as a microbial preparation of a large number of cells of at least one strain to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process. The group of lactic acid bacteria play a essential role in this process, through the production of organic acids (which cause rapid acidification of the raw material), ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes. In this way they enhance the shelf life and microbial safety, improve the texture, and contribute to the pleasant sensory profile of the end product.

Nonfermented probiotic milks also exist in Europe, although they do not have a long history of traditional use; many of them are available only at regional level and they have a moderate success on the market. Probiotics can also be included in juices and berry soups, ice cream, cheese, candy and chewing gum, although these do not play a major role in the marketplace. Sometimes, probiotics are combined with soy and oat and then they are added to cow milk-based probiotic products. Alternatively, entirely oat- or soy-based material is fermented with probiotics and other cultures. A new and promising product category includes breast-milk substitutes, milks for older babies (i.e. “follow-on” milks), and special infant formulas. The legislation for this kind of products is extremely strict and the strains used must be well documented.

In table 4 and 5 the most common species used in probiotic foods and food supplements are listed.

Table 4. The most common species used in probiotic foods

Lactobacillus acidophilus	*Lactobacillus johnsonii/gasseri*
Lactobacillus casei	*Lactobacillus paracasei*
Lactobacillus rhamnosus	*Lactobacillus plantarum*
Lactobacillus reuteri	*Bifidobacteriumj animalis/lactis*
Bifidobacterium bifidum	*Bifidobacterium breve*
Bifidobacterium longum	*Bifidobacterium adolescentis*

Table 5. The most common species used in probiotic food supplements

Lactobacillus acidophilus/johnsonii/gasseri
Lactobacillus casei
Lactobacillus paracasei
Lactobacillus rhamnosus
Lactobacillus plantarum
Lactobacillus reuteri
Bifidobacteriumj animalis/lactis
Bifidobacterium bifidum
Bifidobacterium breve
Bifidobacterium longum
Bifidobacterium adolescentis
Streptococcus thermophilus
Lactobacillus delbrueckii subsp. *bulgaricus*
Enterococcus faecalis
Enterococcus faecium
Bacillus subtilis
Bacillus clausii
Escherichia coli strain Nissle
Saccharomyces boulardii and other yeasts

A wide variety of probiotic strains is used, either singly or in combination, as supplements in several product formulations: hard gelatin or vegetable capsules, tablets with or without enterocoating, chewable tablets, and sachets (Saxelin, 2008).

Some industrial applications of probiotics are listed below.

1. Food preservation and safety

Lactic acid bacteria strains represent an alternative to the use of chemical additives (such as nitrite, sulphite, propionic acid, sorbic acid and benzoic acid) used in food preservation, because of the production of several natural antimicrobials, including organic acids, carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, reuterin and reutericyclin, which help to contrast microbial contamination.

The in situ production of bacteriocins may increase the competitiveness of the producer strain in the food matrix and contribute to the prevention of food spoilage. For instance, bacteriocin-producing LAB can be used as an alternative to potassium nitrate to prevent the contamination of cheese by clostridia (Thomas et al., 2000). Another example is the suppression of flavour-disturbing contaminating microbes, e.g., certain strains of *L. lactis* which produce off-flavours in dairy products (Stanley, 1998). In addition, many bacteriocins are active towards foodborne pathogens such as *Clostridium botulinum*, *Staphylococcus aureus*, and *Listeria monocytogenes*. Several studies have indicated that LAB starter strains are able to produce their bacteriocins in food matrices and consequently show an inhibitory activity towards sensitive food spoilage or pathogenic bacterial strains.

The reuterin (β-hydroxypropionaldehyde) produced by *Lactobacillus reuteri* is active towards a wide spectrum of bacteria, moulds and yeasts, but it is not formed in sufficient amounts in the presence of sugars. Reutericyclin, a tetramic acid antibiotic with broad antimicrobial activity produced by *L. reuteri*, is believed to be responsible for the stability of certain German sourdoughs (Messens and De Vuyst, 2002).

2. Probiotics for a more appealing product

Improvement of texture

In order to give a desired texture and mouthfeel to yoghurt, skim-milk powder, whey, gelatine (e.g., starch, pectin, guar gum, and alginate) and microbial polysaccharides (e.g., xanthan and gellan) are frequently added to the milk and this represents an extra cost for the producer. Polysaccharides increase the viscosity and firmness, improve the texture, reduce the susceptibility to syneresis, and contribute to the mouthfeel of low-fat products. LAB are able to product natural texture-improving sugar polymers (exopolysaccharides) for the manufacturing of yoghurts, sour cream and whipped toppings, ice cream, and of low-fat Mozzarella (Leroy and De Vuyst, 2004) through the in situ use of functional starter cultures. Another application can be found in the bakery industry for a beneficial effect on bread volume and staling (Tieking et al., 2003). The biodiversity of exopolysaccharides produced by LAB from artisan yoghurts, fermented milks, vegetables, and cerealsis being investigated together with the conditions for an optimal production, and their technological implementation in the industrial production of fermented foods.

Another example of texture improvement of foods through functional starter cultures is the use of amylase-producing LAB. LAB producing thermostable amylases can help cereal fermentations, in particular in the sourdough technology for the natural inhibition of staling in bread.

Production of aroma and flavour

LAB contribute to the aroma and flavour of fermented products. Their action of acidification on the food often produces a tangy lactic acid taste and frequently exerts proteolytic and lipolytic activities and produces aromatic compounds from, for instance, amino acids upon further bioconversion. The control over the activities of peptidases from LAB is a key target of the cheese ripening technology. For instance,

Italian ewe milk cheeses are characterised by a very heterogeneous non-starter lactic acid bacterial flora which is influenced by geographical and technological factors, and which could be responsible for cheese diversity (De Angelis et al., 2001). Such strains offer an important base for product innovation, consequently research is going on to study their application in the food fermentation industry. The addition of NSLAB as adjunct cultures for cheese manufacturing increases. The level of free amino acids, peptides, and free fatty acids is increased adding NSLAB as adjunct cultures for cheese manufactoring; this often leads to the intensification of flavour and accelerated cheese ripening and they help to reproduce the flavour of raw milk cheeses when pasteurised milk is used (De Angelis et al., 2001).

Homofermentative LAB convert the available energy source (sugar) almost completely into lactic acid via pyruvate to produce energy and to equilibrate the redox balance. However, pyruvate can lead to the generation of many other metabolites such as acetate, ethanol, diacetyl, and acetaldehyde. In this way, LAB produce volatile substances that contribute to the typical flavour of certain fermented products, such as sourdough (determined by the lactate/acetate ratio), kefir and koumiss (ethanol), butter and buttermilk (diacetyl), and yoghurt (acetaldehyde). The control of optimal fermentation leads to the improved production of some of these volatiles whereas the metabolic engineering focuses on the steering of the metabolic flux in a well-defined direction. Strategies aiming at a direct modification of the redox balance have led to overproduction of the desired metabolites mentioned above (Leroy and De Vuyst, 2004). Alternatively, the introduction of novel enzymatic activities into LAB may result in the formation of cells which produce interesting metabolites from the supplemented sugar. The overproduction of alanine dehydrogenase in suitable *L. lactis* cells has led to a homofermentative, stereospecific production of L-alanine from pyruvate (Hols et al., 1999). L-Alanine is used as a sweetener in the food industry and its in situ production can lead to dairy products with an intrinsic sweetness.

3. Functional starters for the acceleration of the maturation process of cheese

Several aromatic compounds are generated during the maturation of cheese due to the action of endogenous milk enzymes and to the proteolytic and lipolytic activities of LAB present in the cheese. The maturation stage is time- and space-consuming so accelerated maturation techniques are under study. Besides the rational selection of the LAB starter and co-cultures and the application of process conditions for optimal activity of the endogenous enzymes, the addition of exogenous enzymes (enzyme-modified cheese) as well as the increased in situ autolysis of the LAB represent alternative solutions. The autolysis of the starter cells is followed by the release of intracellular peptidases in the curd. Bacteriocins may cause bacteriolysis, for example by inducing autolysins or by deregulating the enzyme action due to energetic deficiency, which will result in the degradation of the cell wall and the consequent cell lysis.

4. Functional starters with a health advantage: production of nutraceuticals and reduction of toxic or antinutritive factors

Nutraceuticals are food components that can contribute to the health of the consumer through a specific physiologiacl action.. Several nutraceuticals from bacterial origin have been added to food products. The activity of LAB can be modified to increase the content of nutraceuticals in fermented foods such as fermented dairy products by selecting the strain and optimising the process.. As an example, fermented milks can be produced with LAB starter strains which produce high amounts of low-calorie polyols so as to reduce the sugar content (Wisselink et al., 2002). Similarly, the use of oligosaccharide-producing LAB that produce sugar polymers with a controlled structure and chain length (and consequently molecular mass) may yield fermented products with health applications. The health effects of such oligosaccharides are ascribed to their low-calorie character, their fibre-like nature, and their bifidogenic effect. In addition, certain LAB, such as the yoghurt bacteria *Lb. delbrueckii* subsp.

bulgaricus and *S. thermophilus*, are able to produce vitamins such as folate. A controlled use of these bacteria may lead to dairy products with increased folate content. The fermentative action of specific LAB stains may lead to the removal of toxic or antinutritive factors, such as lactose and galactose from fermented milks to prevent lactose intolerance and the accumulation of galactose, or the removal of raffinose, stachyose, and verbascose from soy to prevent flatulence and intestinal cramps, of proteinase inhibitors from legumes and cereals to prevent maldigestion, of phytic acid and tannins from cereals and legumes to increase mineral bioavailability, and of natural toxins such as cyanogenic glucosides from cassava as well as of biogenic amines from traditional fermented foods (Holzapfel, 2002).

Probiotics for farm animals

In the early years of last century a number of human studies were carried out, but only in 1960s probiotics started to be used in the farm industry. In particular, the Swann Committee in 1969 suggested the limitation of the use of antibiotics in animal feeds: their use must be restricted to those antibiotics not used therapeutically. This fact stimulated the research on probiotic bacteria. Today, the probiotic research is applied to pets, horses and other farm animals, while the majority of research is done in chickens and pigs (Musa et al., 2009).

In the animal gut there is a very complex population of micro organisms which interact with each other and with the host animal. Estimates rate the number of different types of micro organisms in the gut at 400 and the total number of bacterial cells at 10^{14}. Although the composition of the gut microflora is fairly constant and characteristic for each host species, it can be affected by various factors such as:

- age: the microflora of live young suckling mammal is different from that of the adult

- diet: to some extent this will be responsible for the changes seen with age, but even among adults the composition of the diet can affect the composition of the gut microflora
- environment: the conditions under which farm animals are reared differ from the natural conditions under which their wild counterparts develop. The physiological responses to the artificial nature of the domestic/farm environment may affect the gut microflora
- stress: the unnatural conditions of farm rearing produce stresses which induce hormonal changes which can, in their turn, affect the mucous secretion and the flora composition of the gut.
- medication: the use of antibiotics and other chemical antibacterial compounds either as growth promoters or as therapeutic agents can change the gut microflora in such a way to allow the growth of pathogens.

Probiotic preparations can contain either only one strain of microorganism (e.g. *Lactobacillus reuteri* in GAIA feed) or different strains (e.g. Protexin which contains *L.acidophilus, L. rhamnosus, L. plantarum. L. delbrueckii* subsp. *bulgaricus, S. thermophilus, Enterococcus faecium, Bifidobacterium bifidum, Candida pintolopesii* and *Aspergillus oryzae*). This latter type of preparation can be justified by the claim that it works in a broad spectrum and can be expected to be active in different species of host animal and against different conditions, such as microbial infections and antibiotic-relieved growth depression. The most important probiotic microorganisms used for farm animals are listed in table 6. The main targets of animal probiotic preparations are chicken, pigs and cattle, but the efficacy of probiotics has been demonstrated also in pets, horses and other farm animals (Fuller, 1999).

Table 6. Microrganisms used in probiotics for farm animals

Lactobacillus acidophilus	*Bifidobacterium pseudolongum*
Lactobacillus rhamnosus	*Bifidobacterium brevis*
Lactobacillus casei subsp. *casei*	*Bifidobacterium bifidum*
Lactobacillus reuteri	*Bacillus subtilis*
Lactobacillus plantarum	*Bacillus cereus*
Lactobacillus fermentum	*Bacillus toyoi*
Lactobacillus delbrueckii subsp. *bulgaricus*	*Bacillus natto*
Lactobacillus brevis	*Bacillus mesentericus*
Lactobacillus helveticus	*Bacillus licheniformis*
Lactococcus lactis	*Clostridium butyricum*
Streptococcus salivarius subsp. *thermophilus*	*Pediococcus pentosaceus*
Streptococcus lactis	*Saccharomyces cerevisiae*
Enterococcus faecium	*Candida pintolopesii*
Enterococcus faecalis	*Aspergillus oryzae*

Many authors suggest the importance of the choice of the right strain and the dose on the results of the probiotic administration in animal, but it seems important also to take into consideration the age of the animal, his health, the different composition of the probiotic preparation, the conditions of management of the animals and other environmental factors. Fuller (1999) remarked that “if the right probiotic is given at the right time in the right dose, it will have a significant effect on the animal’s health and/or growth which will be reflected as a significant improvement in the farmer’s bank balance”.

Modern rearing methods which include unnatural rearing conditions and diets induce stress and can cause changes in the composition of the microflora which compromise the animals' resistance to infection. The aim of the probiotic approach is to repair the deficiencies in the microflora and restore the animals' resistance to diseases. Such a treatment does not introduce any external chemicals into the animal's internal

environment and does not run the risk of contaminating the carcass and introducing hazardous chemicals into the food chain.

Probiotics are now replacing the chemical growth promoters for farm animals and claims have also been made for their ability to increase **r**esistance to diseases. The benefits claimed for probiotics in farm animals are as follows:

- Increased growth rate
- Improved digestion
- Provision of essential nutrients
- Greater resistance to infectious diseases
- Improved feed conversion
- Better absorption of nutrient
- Increased egg production
- Increased egg quality
- Improved meat quality and less contamination
- Improved milk yield
- Improved milk quality
- Reduced morbidity or mortality on animals

It has been shown that the gut microflora is involved in the protection against a variety of pathogens including *Escherichia coli, Salmonella, Campylobacter* and *Clostridium*.

Consequently the probiotic approach may be effective in the prevention and therapy of these infections.

The selection of the organisms studied has been largely empirical but is has been established that obligate and facultative anaerobes are required. Mead concluded that the most active organisms in protecting chicks were the lactobacilli (*L. acidophilus, Lactobacillus fermentum, Lactobacillus salivarius*) and certain gram-positive anaerobic cocci (Mead et al., 1987). The mixtures tested ranged from 10-50 different

strains. The method of administration was also examined; oral dosing proved the best but not commercially applicable. Instead, the spraying of the eggs and injection into the air sac gave encouraging results.

The use of probiotics as farm animal feed supplements

The use of probiotics as farm animal feed supplements started in the 1970s. They were originally incorporated into feed to enhance the animal's growth and to improve its health with an increased resistance to diseases. The effect of probiotics was thought to be on the gastrointestinal tract and could affect the incidence of diarrhoea and other gut infections. However, recent studies in several countries have shown that the effects may be more general. According to the results obtained, some of the bacteria used in probiotics (lactobacilli) are capable of stimulating the immune system. This opens up a whole new area of potential application for probiotics in which it will be possible to influence disease situations in sites remote from the gut, and also prevent intestinal disease. There are two ways in which the probiotic microorganisms in the gut can stimulate the immune system: they can migrate through the gut wall as viable cells and multiply to a limited extent, alternatively antigens released by the dead organisms can be absorbed and stimulate the immune system directly. A third way could be represented by the indirect action of the lactobacilli through an effect on the other components of the gut flora.The product of this change will induce the immune response.

Animal trials

A typical example of animal trial is done in Spain by Tortuero et al. (1995). They compared two probiotic preparations containing a) *Enteroccus faecium* and *Lactobacillus casei* and b) the two yoghurt starter organisms, *Streptococcus salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. In the first experiment the preparation containing *E.faecium* and *L. casei* increased the weight gain during the experimental period up to 21 days of age. The yoghurt-starter cultures

also gave a positive response but only between 12 and 21 days of age. However, when the preparation containing *E. faecium* and *L. casei* was retested, it failed to improve the weight gain. The second test was carried out with pigs from split litters, whereas the first experiment was carried out with piglets distributed randomly in the two treatments. At the moment it is difficult to assess the significance of this and undoubtedly there were other factors which varied between the two trials. This result confirms that under the right conditions a significant growth response can be obtained. But it also clearly shows the kind of confusion existing in this type of experimentation in which attempts to repeat a positive result often fail and it is impossible to explain such a failure since it is difficult to ensure that all the relevant factors other than the supplementation are constant. There was a larger decrease in the coliform count from log10 8.10 down to 6.85 in the group, showing an improvement in the growth rate. This group also showed an increase in the concentration of interleukin Z (from 3.74 ng/g down to 7.43 ng.g). Neither of these differences were statistically significant. However, they do agree with other studies showing affects on the coliform count and the immune status.

An extensive and well conducted trial on poultry was published in 1996 (Nahashon et al. 1996). It considered a wide range of features including growth rate, feed conversion and egg production. The feed supplement employed was a *Lactobacillus* strain but no further information regarding the identification of the species was given. The trial looked at the effects occurring during the pullet phase (7-19 weeks) and during the egg-laying phase (20-59 weeks). During the pullet phase, the feed consumption and the weight gain increased as a result of feeding the *Lactobacillus* supplement, but there was no weight gain for the layers. However, there was an increased daily feed consumption and an increased egg size.

The quality of the eggs was not affected. In the past, several studies claimed positive effects on egg production but they were not statistically significant. An interesting new approach to probiotic administration to chickens has appeared recently. Embrex, in the States, developed a device for inoculating eggs with vaccine. In collaboration

with Probiotics International Ltd, UK, trials were carried out with the multistrain probiotic, Protexin. Eggs at 18 days incubation were inoculated with Protexin into the airsac or amnion. This procedure had no effect on hatchability; in fact, the injected eggs had a slightly increased hatchability and accelerated hatch date. The results so far available are only very preliminary, but the mean figures from the two trials show that there is an increase in body weight at 2 weeks of up to 8.7% depending on the dose and the rate of inoculation.

The work on probiotics for cattle has increased in recent years. In calves, studies have been carried out using *Saccharomyces cerevisiae*, *Aspergillus oryzae*, various species of *Lactobacillus* and *Enterococcus faecium*. In the past ten years, positive effects (although not always significant) on feed intake, weight gain, earlier weaning, reduced scouring decreased faecal coliform count and a lower demand for antibiotic treatment have been found. It is interesting to note that benefits don't have to be always measurable in terms of increased growth rate or feed efficiency; as in the case of a study by Seymour et al. (1995), the effect may be demonstrated by monitoring the days of fever experienced by the animal and the number of antibiotic treatments required to maintain it in good health. In adult cattle the studies have used mainly the fungal probiotics. With this preparation for beef cattle, recent experiments have shown improvements in feed efficiency and dry matter intake. The analysis of all the published data on this subject indicates that the average increase in daily gain of cattle fed yeast culture was 7.3%. The corresponding figure for feed efficiency was 6.0% (Huber, 1990). Numerous studies have been done with lactating cattle. Over several years the average increase in milk yield of cows treated with *Aspergillus oryzae* has been of 2.5%. Increases in milk yield have also been obtained by supplementation with yeast. Both types of supplementation have induced higher butterfat concentrations in milk.

Campylobacter jejuni

Campylobacter jejuni is a pathogenic bacteria recognised as the most frequent cause of gastrointestinal disease in industrialised and developing countries. The most common route of infection is by ingestion of contaminated food, milk or water, with poultry being a particularly common source of contamination, as *C. jejuni* is a commensal of the gastrointestinal tract of many species of birds. Therefore, the consumption of poultry meat contaminated by *Campylobacter* is a significant risk factor /which can cause infection .

The use of antibiotics in feed to prevent colonization of *Campylobacter* has been prohibited in Western Europe so researches should be carried out to identify alternative strategies to contrast the infection from this bacterium in animals . In addition, the common trend to prescribe antibiotics has favored the emergence of antibiotic-resistant pathogen strains often in association with the disruption of protective flora and raising the risk of pathogen infections. As a consequence, there is an increasing interest in the development of adjunctive or alternative therapies based on bacterial replacement through the use of probiotics derived from the natural intestinal flora. Probiotic bacteria are useful in the management of gastrointestinal infections in both human and animals (Salminen et al., 1999). The possible mechanisms underlying these inhibitory effects include competition for nutrient and adhesion sites, toxin inactivation, secretion of antimicrobial substances, and immune stimulation, but their respective roles remain unclear (Fooks et al., 1999).

Chapter 5. *In vitro* selection of probiotic strains

Although progress in probiotic research has been achieved over the past few years, not all of the available probiotic bacteria which are on the market have adequate scientific documentation (Sanders and Huis in't Veld, 1999). If nutritional and health benefits are to be derived from products containing probiotic bacteria, it should be desirable to understand the underlying mechanisms , and to use the strains that have proved to be the most promising. The probiotic concept will only gain acceptance if these underlying mechanisms are elucidated. Consequently, it is necessary to establish rational criteria for the screening and selection of candidate microorganisms and also to evaluate the efficacy of the selected strains or the food products in well-controlled human clinical trials.

Since there was no international consensus on the methodology to assess efficiency and safety of probiotics, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have compiled and evaluated the scientific evidence on functional and safety aspects of probiotics and generated "Guidelines for the Evaluation of Probiotics in Food" during the joint workshop group held in Canada, in May 2002 (Fao and WHO, 2002). FAO and WHO and the countries they represented requested guidelines and recommendations for the criteria and methodologies required to identify and define probiotics and establish the minimum requirements needed to accurately substantiate health claims. Although the FAO and WHO reports focused on foods, many of the recommendations, including the definition of probiotics, were approved at the Meeting of the International Scientific Association

for Probiotics and Prebiotics in May 2002. A scheme outlining the guidelines for the evaluation of probiotics is shown in Fig.8 below.

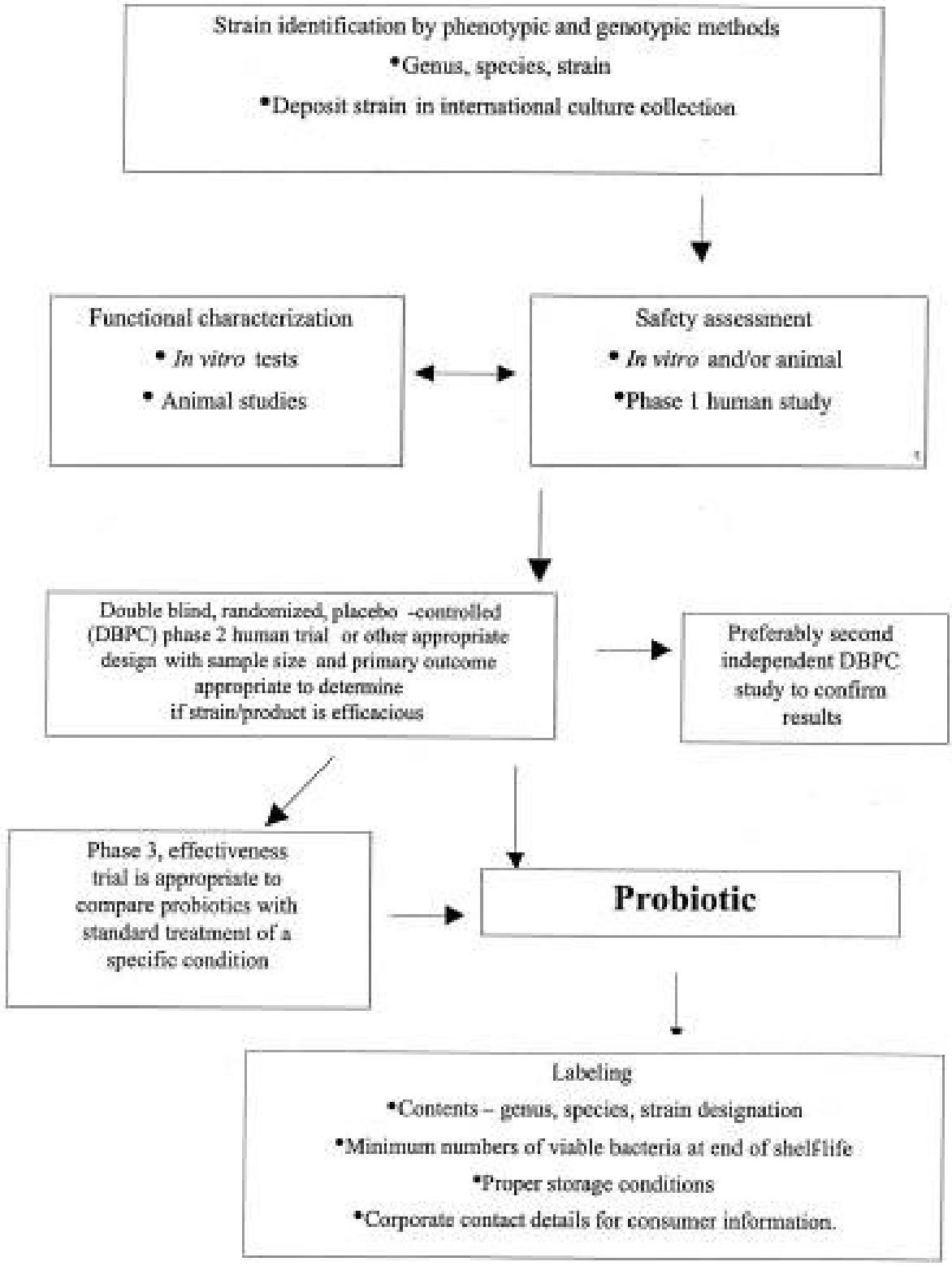


Fig. 8. FAO and WHO guidelines for probiotics in food

These guidelines address the following points:

- Strain identification

The first consideration is to identify and characterize the organism at the genus and species-level. A combination of phenotypic and genetic tests should be used. Once the strain has been identified, a scientifically recognized name must be employed and the strains must be deposited in an internationally recognized culture collection.

- *In vitro* tests to screen potential probiotics and assessment of efficacy

In vitro tests of candidate probiotic strains, some of them summarized in Tab. 7, are thought to provide some insight for a more appropriate choice for *in vivo* functionality.

Tab 7. Main in vitro tests currently used for the study of probiotic strains (Modified from report FAO, 2002)

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

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

The transit of probiotics included in foods through different sections on the GIT takes variable time and it is submitted to different stressful conditions. In human, after

mastication, the first barrier that bacteria must overcome is the low pH value of the stomach with values ranging from 1 to 3 and mean exposure time of 90 minutes. In the duodenum the pH value rises to 6-6.5, but bile salts are poured from the gallbladder and reach concentrations ranging from 1. to 2% during the first hour of digestion which decrease afterwards to 0.3% w/v or lower (Lee and Salminen, 2009). The residence period in the small intestine until 50% emptied varies between 2.5 and 3 hours and the transit through the colon could take up to 40 hours. In this location pH values are close to neutral (from 5.5 to 7) and the physiological concentration of bile salts is lower.

In animal the pH values and bile salts concentrations are different. For the screening of putative probiotic bacteria researchers simulate the GIT conditons in vitro, testing several pH values and bile concentrations for variables times in order to determine the survival of the strain under test.

In the complex GIT ecosystem probiotics have developed mechanisms to survive in competition with other microorganisms. Essentially, the antagonism is exerted by competition for nutrients and for physical location, but also through the production of antimicrobial substances: the ability of probiotics to produce antimicrobials is one mechanism to inhibit, exclude or compete with adherent enteropathogens for the ecological niche. The inhibition ability is strain and culture condition-dependent and several molecules and mechanisms are involved in the interrelationship between probiotics and pathogens.

Even if LAB strains have a long history of safe consumption in traditionally fermented products and several species have been awarded a GRAS status by the American Food and Drug Association or a qualified presumption of safety (QPS) consideration by the European Food Safety Authority (EFSA), some characteristics must be carefully studied to ensure the safety of the novel lactobacilli and bifidobacteria strains.

- Safety

As efficacy is inextricably linked to safety, any claims of health benefits for a probiotic require substantiation by scientific evidence. The employment of "history of safe use" as a criterion for the safety of food organisms is an arbitrary classification. Lactic acid bacteria and yeasts intrinsic to the production of traditional foods have been accepted as safe without any real scientific criteria, partly because they are normal commensal flora, and partly because they have been consumed through centuries presumably without adverse effect. The shift from dairy foods to complementary or prescribed medicines with therapeutic claims has elevated probiotics to a class that was once the exclusive field of pharmaceuticals. How to assess the safety of new probiotic products needs to be re-evaluated in these altered circumstances. Regulators must judge whether a probiotic is a food, a supplement or a clinical therapy and develop enforceable safety standards accordingly. If probiotics are intended for therapeutic use they must be evaluated for quality, safety, and efficacy in the same way as any other therapeutics-with documented and verifiable characterization of the active ingredient, dose, efficacy, safety, and adverse effects (Lee and Salminen, 2009). For the Evaluation of Probiotic in Food, the FAO and WHO workshop in the meeting held in Canada (FAO and WHO, 2002) advised to characterise probiotic strains at least with the following tests: determination of antibiotic resistance profile, assessment of metabolic activities, assessment of side effects during human studies and postmarket epidemiological surveillance of adverse incidents in consumers.

In addition, if the strain under evaluation belongs to a species known to produce a mammalian toxin or to have haemolytic potential, it must be tested for these characteristics.

The EFSA has proposed a scheme based on the concept of QPS, defined as "an assumption based on reasonable evidence" which has allowed the application of certain restrictions (EFSA, 2005). The scheme aims at achieving a consistent generic safety assessment of microorganisms through the food chain without compromising

safety standards. Individual evaluations would be limited to aspects particular to the organism, such as acquired antibiotic resistance determinants in LAB.

Bernardeau and collaborators (Bernardeau et al., 2008) consider this generic approach to the safety assessment of microorganisms not relevant to the *Lactobacillus* genus and have proposed modifications. They claim that LAB are not a homogeneous group as some species are pathogens, and that the rarely pathogenic *Lactobacillus* genus should undergo its own limited safety assessment. The genus should be awarded the status of Long Standing Presumption of Safety based on its long history of safe use in fermented foods. Individual species could then be assessed for safety based on one, two, or a full suite of tests, depending on the intended use. The first safety test would be to demonstrate an absence of antibiotic resistance and the ability for transference. The second, a high dose tolerance test in animals, would be required if the organism was not resistant to antibiotics and was a known lactobacillus for which a new application was being proposed. A full safety assessment would be required if the body of knowledge was insufficient.

Selection of probiotic strains for use in food and animal feed

Food companies worldwide are seeking ways to incorporate probiotics into a much broader range of foods and beverages. However, incorporating live probiotic microorganisms into foods and keeping them alive throughout shelf life is a significant challenge for food technologists.

For a successful delivery in foods, probiotics must survive food processing and storage during maturation and shelf-life. To incorporate probiotics into foods companies have to take into account several parameters. The critical points to address when incorporating probiotics into foods (Lee and Salminen, 2009) include:

- The selection of a compatible probiotic strain/food type combination
- The use of food-processing conditions that are compatible with probiotic survival

- If fermentation is required, ensuring that the food matrix will support probiotic growth
- The selection of the product formulation, packaging and environmental conditions to ensure adequate probiotic survival over the product's supply chain and during shelf storage.
- Ensuring that addition of the probiotic does not adversely impact on the taste and texture of the product.

Acid stress

The growth of LAB is marked by the generation of lactic acid as end product of fermentation and this accumulates in the extracellular environment. This implies that LAB often confront with acid stress and acid is an important environmental stress present in LAB during the fermentation of foods and beverages. It must be noted that lactic acid is a weak organic acid that it does not charge at low pH and can easily pass the cell membrane in the protonated form. The marked production of organic acid from these bacteria creates a hostile environment for many other organisms. Many methods of food preservation by fermentation are based on this characteristic. These bacteria can also face an acidic environment in the stomach after consumption, and the development of probiotics revived the interest for studies about LAB survival in the digestive tract. The cariogenicity of oral LAB such as streptococci and lactobacilli is directly linked to their acidogenicity (i.e. the ability to produce acid at low pH) and acidurance (i.e. the capacity of functioning at low pH). With the only exception of some species of the genera *Lactobacillus*, *Leuconostoc* and *Oenococcus*, LAB are neutrophiles (i.e., optimal pH for growth between 5 and 9). Not much is known about the effects of acid stress on bacterial physiology. It is well established, however, that acids can passively diffuse through the cell membrane and after access the cytoplasm and rapidly dissociate into protons and charged derivatives to which the cell membrane is impermeable. The intracellular accumulation of protons may have the

effect of lowering the intracellular pH and thus affect the transmembrane ΔpH which contributes to the proton motive force used as an energy source in numerous transmembrane transport processes. The activity of acid-sensitive enzymes is also reduced and proteins and DNA are damaged by the internal acidification. There is also a detrimental effect of the accumulation in the cytoplasm of the anionic moiety on cellular physiology possibly through a chelating interaction with essential elements.

In LAB, acid tolerance rises in at least two distinct physiological states:

1. during logarithmic growth an adaptative response can be induced by incubation at a non-lethal acidic pH
2. after reaching the stationary phase, acid tolerance increases as a result of the induction of a general stress response

The latter response is usually independent of the external pH. It is not known whether these responses are independent or may overlap. The growth in biofilms may be a third state which improves the acid tolerance, but it has been only demonstrated for *S. mutans* (Li et al., 2001; Zhu et al., 2001).

Bile salts stress

Bile is a complex digestive secretion which helps the dispersion and absorption of fats. Bile acids (also often called bile salts) are the major constituents of bile and derive from cholic acid (CA) which is itself synthesized from cholesterol. In the liver some bile acids are conjugated to glycine or taurine.

The bile acids undergo extensive chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) in the colon almost solely as a result of microbial activity. The possible toxic effects of bile acids for bacterial cells is still to be fully understood; however, as they act as detergent and dissemble biological membranes, bile acids are surface active, amphipatic molecules with potent antimicrobial activity. Gram-positive bacteria do not have an outer membrane

which can constitute the first permeability barrier, so they are often more sensitive to the toxic agents in their environment than Gram-negative bacteria. Both Gram-negative and Gram-positive bacteria develop specific mechanisms to resist the toxic action of bile and manage to live in a bile-rich environment such as the digestive tract. The resistance to bile stress has been mostly examined in two categories of bacteria: the enteric pathogens, which can survive in the digestive tract, and the food-associated LAB or bifidobacteria considered as potential probiotics and often selected to resist to the digestive stress (Van de Gutche et al, 2002).

Bile acids can be metabolized by some microorganisms of the intestinal flora including some lactic acid bacteria and it is believed that this ability can contribute to the protection against bile. In lactobacilli, the bile salt hydrolases (BSH) deconjugate bile acids, hydrolysing the amino acid glycine or taurine from the steroid core. This hydrolysis modifies the properties of bile and remarkably reduces its solubility at low pH and its detergent activity. Some studies have reported an active uptake of CA, dependent on the pH and the presence of glucose in several species of lactobacilli. This activity, not related to BSH, results in an intracellular accumulation of CA. Mot much is known about the role of this system in lactobacilli and its relation to CA or bile tolerance.

Choice of a right combination between probiotic organism and food

When incorporating a probiotic into food the first step is to identify the compatibility between the attributes of the selected strains and the food production steps and food matrix. The difference in the technological characteristics of different probiotic species and strains requires a special care to be taken in selecting the most appropriate strain for a particular application. This may involve a compromise between the desired health attributes and the technological capabilities of particular strains for particular food applications. When developing new products some research may be required to ensure that the selected strain is able to survive well in the food,

provided the appropriate technological properties and that the added probiotic does not adversely affect the taste, the texture and the smell of the food or beverage. The metabolism of the probiotic organism is an important consideration in fermented probiotic foods, not only for the growth and survival of the probiobic, but also for food quality. For example, heterofermentative lactobacilli that produce CO_2 as a metabolic end product are not suitable where gas formation adversely impacts on food quality.

Physiology of the probiotic

An important factor in probiotic survival is the physiology of the bacteria when prepared, and the physiological state of the bacteria in the product itself: if the food product is dry, i.e. a powdered infant formula, the probiotic will also be dried and in a quiescent state during storage. However, when included in a wet product (like a yogurt), the bacteria will be in a vegetative state and potentially metabolically active. Bacteria are able to respond to stressful environments through the induction of various stress tolerance mechanisms. The induction of stress proteins by exposure of the cells to sublethal stresses such as heat, cold, starvation, low pH, and osmotic stress can condition probiotics to better tolerate environmental stresses in food production, storage, and gastrointestinal transit.

Technological characteristics of the probiotic:

Temperature

One of the functional aspects for the characterisation of novel putative probiotic strain for use in food is temperature (see fig. 9). Temperature is a critical factor influencing probiotic survival during manufacture and storage: the lower the temperature the stabler the probiotic viability in the food product . During processing,

temperatures above 45-50°C will be detrimental to the probiotic survival. The higher the temperature, the shorter the period of exposure necessary to severely decrease the numbers of viable bacteria, ranging from hours or minutes at 45-55°C to second at higher temperatures. Elevated temperatures also have a detrimental effect on the stability during the shipping and storageof the product.

Protein denaturation is the greatest effect induced by high temperature; however, studies have identified membranes and nucleic acids as cellular sites of heat injury. The heat stress also affects the transmembrane proton gradient, resulting in a decrease of the intracellular pH.

During the exposure of cells to heat shock, the responses take place through the increased synthesis of a group of evolutionary conserved heat shock proteins which promote the correct folding of nascent polypeptides, the assembly of protein complexes, the degradation and translocation of proteins. Heat stress in lactobacilli has been studied by analyzing its effect on growth, heat tolerance and protein synthesis. The genetic differences among species, the physiology of the cells and other environmental factors (growth medium, pH, water activity, salt content and preservatives) affect the resistance of lactobacilli to heat stress.

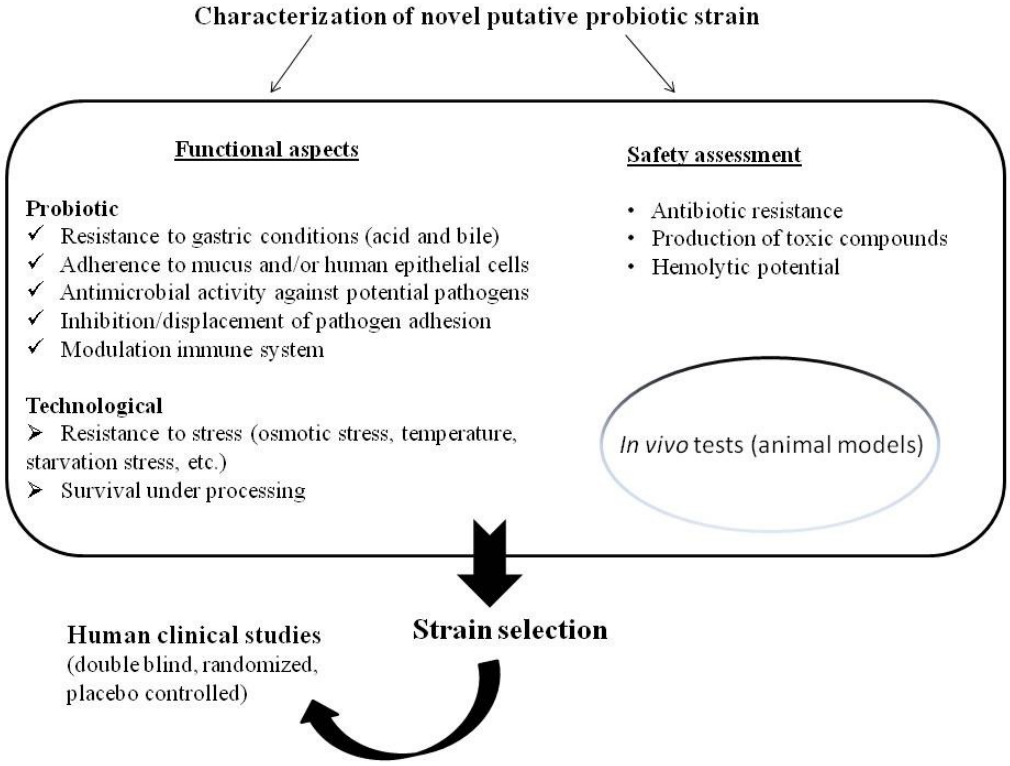


Fig. 9 Procedure for the characterisation of novel strain with putative probiotic status.

Starvation stress

Bacteria spend most of their time in stationary phase. The growth arrest and the onset of the stationary phase can be caused by numerous stress conditions like cold, heat, osmotic, oxidative or acid stress, or starvation. Nutrient starvation is one of the most frequent stresses and the bacterial growth itself contributes to the nutrient exhaustion and the subsequent starvation for one or several compounds. Furthermore, extreme environmental stress conditions may determine a deprivation of one or several components, apart from their direct effects on the cells constituents. For example, extreme acidic conditions can reduce the activity of some transporters and diminish the availability of essential substrates thus provoking indirectly starvation or energy depletion, regardless of the extracellular amount of the substrate (Konings et al. 1997). These conditions of energy or essential elements depletion could be dangerous for long-term cell viability. However many bacteria seem to be well adapted to survive long-term starvation and some can enter a stress-resistant spore-forming process. Others, including LAB, do not show this capacity and have developed other strategies. In these bacteria, nutrient starvation leading to growth arrest is generally linked to the modification of cell morphology, like cell division at the beginning of the stationary phase which leads to a reduction of cell size, as it has been described for many non-spore forming bacteria and also for some enterococci and lactococci. LAB are a heterogeneous group of bacteria which grow in very different media, and therefore do not face identical starvation conditions. That is why, diverse starvation surviving mechanisms developed by different LAB may be observed.

Water activity

For quiescent, dried, probiotic bacteria water activity is a crucial determinant of survival in food products during storage. As moisture levels and water activity increase the survival of probiotics substantially decreases. Probiotics can survive well over long shelf lives (12 months or more) at room temperatures in dried products as long as the low moisture levels in the products can be maintained. In general, the lower the water activity, the better the bacterial survival. There is a substantial interaction between water activity and temperature in relation to their impact on the survival of quiescent probiotics. As the storage temperature is increased the detrimental impact of moisture is magnified. Although the precise mechanisms of cell death remain unclear, osmotic stress appears to play a role, with the presence of smaller molecules resulting in a poorer bacterial survival.

Osmotic stress

Active metabolism takes place if the intracellular conditions remain relatively constant in relation to the ionic composition, pH, and metabolite levels. Moreover, the maintenance of a constant positive turgor is generally considered as the driving force for the cell expansion. The bacterial cytoplasmic membrane is permeable to water but forms an effective barrier for most solutes, consequently a change in the osmolality of the environment could rapidly compromise essential cell functions, and bacteria would have to adapt to such a change in their environment in order to survive. Usually, they can do so by accumulating compatible solutes (by uptake or synthesis) under hyperosmotic conditions, and releasing (or degrading) them under hypoosmotic conditions. Apart from their effect on the osmotic balance, compatible solutes may also stabilize enzymes and thereby provide protection not only against osmotic stress but also against high temperature, freeze-thawing and drying (Poolman et al. 1998).

Viability in probiotic products

In order to have a positive effect on health, probiotics in food should remain viable during the storage and the gastrointestinal transit. Different minimum levels of probiotics in probiotic products have been reported; however these levels are likely to depend on the specific strain used. Dose-response studies are needed in the assessment of probiotic strains, to determine the effective level of bacteria in one given product.

Antibiotic resistance

About 50 years ago, antibiotics were introduced for the treatment of microbial diseases. Since then, the greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria. The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the increase in the use (over-use and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria.

Between men and animals antibiotic resistant bacteria are mainly trasmitted via the food chain. In particular the animanl indigenous microflora and the human GIT came into contact throug fermented dairy products and fermented meat which haven't been heat treated before consumtion. Although most food-associated lactic acid bacteria have acquired the GRAS status, the potential health risk, due to the transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of the human gastrointestinal tract and hence to the pathogenic bacteria, has not been fully addressed. Fermented milk products use lactic starter cultures, and these bacteria enter our intestines in large numbers; here they interact with the intestinal microflora. The commercial introduction of probiotics containing antibiotic resistance strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens.

The evolution and enrichment of antibiotic resistant bacteria have been recently reported in studies about the application of antibiotics in human and veterinary medicine (Levy, 1997; WHO, 1997), agriculture (Falkiner, 1998) and aquaculture (Reilly and Kaferstein, 1997); the phenomenon is regularly observed when a new antibiotic is introduced (Levy, 1997). Two factors determine the development of antibiotic resistance in bacteria: the presence of resistance genes and the selective pressure by the use of antibiotics (Levy, 1992). Before discussing these two factors,it is necessary to make a distinction between intrinsic and acquired resistance. The resistance to a given antibiotic can be intrinsic to a bacterial species or genus (inherent or natural resistance) and it results in the ability of the organism to thrive in the presence of an antimicrobial agent due to an inherent characteristic of the organism. The intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria. On the contrary, the acquired resistance is present in some strains within a species usually susceptible to the antibiotic under consideration, and might be horizontally spread among bacteria. The acquired resistance to antimicrobial agents can arise either from mutations in the bacterial genome or through the acquisition of additional genes coding for a resistance mechanism. These genetic changes alter the defensive functions of the bacteria by changing the target of the drug, by changing the membrane permeability, by enzymatic inactivation of antibiotic (e.g. by β-lactamases, aminoglycoside acetyl-, nucleotidyl- and phosphoryl-transferases), by active transport of antibiotics (e.g. by membrane inserted ATP-dependent efflux systems), by modification of the target (e.g. methylation of 23S rRNA, mutation of aminoacid sequence of topoisomerase) (Davies, 1997), or by routing metabolic pathways around the disrupted point (Poole, 2002).

The selective pressure imposed by the use of antimicrobial agents plays a key role in the emergence of resistant bacteria. Whenever a mixed bacterial population is exposed to antimicrobial agents, it is likely that there will be bacteria that are resistant to the respective drugs at the concentration applied. Under selective pressure, the

numbers of these will increase and some may pass their resistance genes to other members of the population (Aarestrup, 1999). A single antibiotic may not only be selected for resistance to that particular drug. It can also include resistance to other structurally related compounds of the same class. When antibiotics of different classes share the same target site, and this target site is modified by the product of a resistance gene, cross-resistance between structurally unrelated antibiotics is observed. In addition, a number of plasmids have been identified which carry multiple resistance genes, resulting in co-transfer. The risk of potential spread of resistance genes from probiotics to pathogenic bacteria is therefore higher if the gene is carried on a mobile genetic element (like a plasmid or a thansposon). On the other hand, it is important that a probiotic co-administered with an oral antibiotic therapy is resistant to particular antibiotics.

Regulation

Various regolatory bodies have addressed the safety issues concerning the use of the LAB organisms in animal nutrition in different countries. The presence of acquired antibiotic resistance factors is considered highly undesirable in Europe but of lesser relevance in the USA. In the United States of America, the Food and Drug Administration (FDA) classifies certain microorganisms as GRAS. An organism or a product with a GRAS status is exempt from the statutory premarket approval requirements (Mathur and Singh, 2005). An organism can either be included in the GRAS list or have the status of history of safe use. However, the European view point is more restrictive since the Scientific Committee on Animal Nutrition (SCAN), (one of the main scientific committees in the food and feed area), has put forward the proposal that any listing should be qualified**,** provided that certain specifications are met. This may simply be a requirement to demonstrate the absence of acquired resistance factors, as in the case of many live organisms currently used in manufacturing dairy products or added to them. The development of a system that would allow a qualified presumption of safety (QPS) has been strognly

recommended. In a QPS system the safety assessment of food LAB could be limited to the presence of transmissible antibiotic resistance markers as other tests are not relevant for lactic bacteria. The Opinion of SCAN on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance was adopted on 3 July 2001 and later revised on 18 April 2002 (EuropeanCommission, 2002). According to SCAN all bacterial products intended for use as feed additives must be examined to establish the susceptibility of the component strain(s) to a relevant range of antibiotics. Such tests must be made in a consistent manner using internationally recognized and standardised methods.

The determination of the minimum inhibitory concentration (MIC) of the antibiotic has been suggested along with breakpoints categorizing bacterial species as resistant. It has been mentioned that determination of MIC is not necessary for species designated as inherently resistant to the antibiotic. The general lack of relavant datat hinders the proposal of breakpoints for lactic acid bacteria. For some genera of lactic acid bacteria, such as *Lactobacillus*, there are no generally accepted standard procedures for MIC determination and information on MIC ranges is rather limited. The breakpoints suggested by SCAN (see table 8) may be seen as a practical response to introduce consistency in the separation of strains with acquired transferable resistance form susceptible strains. According to the SCAN**,** every time the MIC value is at or above the given breakpoints further investigation should be carried out.

Table 8. The Microbiological breakpoints used by SCAN categorising bacterial species as resistant (mg/l)

Antibiotic	E.faecium	E.faecalis	Pediococcus	Lactobacillus sp.	Bacillus
Ampicillin	8	8	3	2	2
Streptomycin	1024	1024	32	16	64
Kanamycin/neomycin	1024	1024	32	32	64
Gentamycin	500	500	4	1	8
Chloramphenicol	16	16	16	16	16
Tetracycline	16	16	16	16	16
Erythromycin	4	4	4	4	4
Quinupristin/dalfopristin	4	R	4	4	4
Vancomycin	8	8	R	4	4
Trimethoprim	8	8	16	32	8
Cipro/enrofloxacin	4	2	16	4	1

Linezolid	4	4	4	4	4
Rifampin	4	4	8	32	4

R = Certain species are inherently resistant, and for these species MIC determination is not necessary

In 2005, a new decision of FEEDAP Panel of the EFSA updated anew the criteria used for the assessment of bacteria for resistance to antibiotics of human and veterinary importance (EFSA, 2005). The aim of this decision was to provide guidance for developing studies to show the potential of each bacteria strain to bear resistance and to transfer it**.** The basis of such evaluation started with the determination in vitro of the antibacterial activity (MIC) for a relevant range of antibiotics of human and veterinary importance (table 9). The detection of the MIC above the breakpoint levels in one or more antimicrobials required further investigations to make the distinction between acquired and intrinsic resistance; the microbiological breakpoints categorizing bacteria as resistant are expressed in table aa. According to the principle of FEEDAP, when a bacterial strain proves resistant to a specific antibiotic, while others species are normally susceptible to the same antibiotic, the applicant should evaluate the reason for such resistance. If an acquired resistance may be transferred or if known exogenous resistance genes are present, the probiotic strain is not considered suitable for use as feed additive (Anadon et al., 2006).

In addition, the determination of antibiotic resistance among LAB is affected by problems regarding the use of media and MIC breakpoints for the genera or species; MIC breakpoint values have been shown to be species specific and consequently they vary between species of the same genera (Danielsen and Wind, 2003).

Studiyng the antibiotic resistance of 40 starter cultures and 5 probiotic cultures at the genetic level, Hummel and collaborators in 2007 (Hummel et al., 2007) identified factors likely to hinder the implementation of the safety evaluation scheme proposed in EFSA's QPS system and concluded that there were no approved standard MICs at which an organism may be considered resistant or susceptible to an antibiotic, except for Enterococcus species.

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

Table 9. FEEDAP microbiological breakpoints categorizing bacteria as resistant (mg/l). Strains with MICs higher than the breakpoints below are considered as resistant.

Antibiotic	*Lactobacillus* obligate homofermentative	*Lactobacillus* heterofermetative	*Lactobacillus plantarum*	*Entero-coccus* sp	*Pediococcus*
Ampicillin	4	4	4	8	4
Vancomycin	4	n.r.	n.r.	8	n.r.
Gentamycin	8	8	64	512	4
Kanamycin	16	16	64	1024	4
Streptomycin	16	16	64	1024	4
Neomycin	16	16	32	1024	8
Erythromycin	4	4	4	4	4
Clindamycin	4	4	4	4	4
Quinupristin + Dalfopristin	4	4	4	4	4
Tetracycline	8	8	32	16	4
Chloramphenicol	4	4	8	8	4
Trimethoprim	8	8	8	8	8
Linezolid	4	4	4	4	4

Antibiotic	*Leuconostoc*	*Lactococcus lactis*	*Streptococcus thermophilus*	*Bacillus* spp.	Other Gram-positive
Ampicillin	4	4	4	n.r.	2
Vancomycin	n.r.	4	4	4	4
Gentamycin	4	8	8	4	4
Kanamycin	8	8	8	8	8
Streptomycin	8	16	16	8	8
Neomycin	8	8	8	8	8
Erythromycin	4	4	4	4	4
Clindamycin	4	4	4	4	4
Quinupristin + Dalfopristin	4	4	4	4	4
Tetracycline	4	4	4	8	4
Chloramphenicol	4	8	8	8	4
Trimethoprim	8	n.r.	n.r.	8	8
Linezolid	4	4	4	4	4

Strains with MICs higher than the breakpoints below are considered as resistant.
n.r., not required. including *L. salivarius*.

Aim of the dissertation

Lactic acid bacteria and bifidobacteria are the best candidates for use as protective and probiotic cultures, because they have been used since the beginning of history as starter cultures and they are present in almost all fermented foods like vegetables, meat products and dairy products; in addiction, they are part of the natural microflora of both farm animals and humans and they have a long history of consumption and safe use.

"Protective culture" are bacteria especially selected and developed for their ability to control the growth of pathogenic and/or spoilage microorganisms in fermented food. Their inhibition toward pathogens is due to the direct competition for nutrients and to the production of antimicrobial substances. Protective cultures are also useful in food product, in particular in the extension or mantainance of shelf life by avoiding microbial organoleptic degradation, but also in the reduction of supply chain and distribution costs, in the replacement of chemical preservatives by a natural and safe solution and they are used alone or in conjunction with starter cultures to contribute in formation of texture, colour and flavour of food product. Beneficial bacteria in the food process chain can have a protective role, such as protecting food product/process from the infection or contamination respectively and a probiotic role, conferring a beneficial effect upon the host, either on a farm animal through animal feed, or on the final consumer, through the food product.

The aim of this research is to select probiotic bacteria strains able to inhibit pathogenic bacteria either at the level of food products or at the level of farm animals, that can survive the passage of the gastrointestinal tract and food processing conditions and that can be incorporated in the development of prevention strategies

for foodborne pathogenic microorganisms throughout the food chain. The beneficial effect of the protective and probiotic cultures can take place at any level of the food process chain, from the animals to the food product.

The criteria adopted for the selection of candidate protective and probiotic cultures are in accordance with the relevant international literature and with the guidelines for evaluation of probiotics in food suggested by the Joint FAO/WHO Expert Consultation Report (FAO and WHO, 2002), especially concerning the in vitro functional characterization (resistance to gastric activity, bile acids, antimicrobial activity) and safety assessment (haemolytic activity and antibiotic resistance) of potential probiotics strains. A deeper knowledge of the mechanisms of stress resistance should enable to understand better the bases of the adaptive responses and cross protection, and to optimise their exploitation in order to prepare LAB to be employed in industrial processes and to be administered to farm animals.

PART II

MATERIALS AND METHODS

Chapter 1. Antimicrobial activity

The research described in this thesis is framed within VI FP European Project PathogenCombact (www.pathogencombat.com). The first part of the investigation was carried out on 60 strains of Lactic Acid Bacteria and bifidobacteria provided by DANISCO, a partner of the PathogenCombat Project, and by the BUSCoB collection (Bologna University Scardovi Collection of Bacteria) of the Microbiology area of the Department of Agroenvironmental Science and Technology (University of Bologna, Italy) to assay their antimicrobial activity against *Campylobacter jejuni*.

- *C. jejuni* CIP 70.2 (Type strain)
- *C jejuni* LMG 8842 from the Belgian Co-ordinated Collections of Micro-organisms (BCCM™)
- *C. jejuni* 221/05 wild strain from poultry

The antimicrobial activity was tested in vitro by using the spot agar test, based on the observation of the inhibition of pathogen's growth using live cells and the neutralised free-culture supernatants of strains tested, following the procedure of Schillinger and Lucke (1989). The strains which showed to inhibit the growth of pathogen, were then submitted to the well diffusion assay, performed as described by Casla et al. (1996).

Tab. 10. Bacterial strains used

Strain	Species	Source

PCA 236	*Lactobacillus* sp.	Kasseri cheese
PCA 259	*L. plantarum*	Xynotyri cheese
PCA 263	*Lactobacillus* sp.	Xynotyri cheese
PCA 275	*Lactobacillus* sp.	Feta cheese
PCA 293	*L. plantarum*	Feta cheese
PCD 71	*Lactobacillus* sp.	Unknown (Danisco)
PCD 101	*L. pentosus*	Unknown (Danisco)
PCD 119	*Leuc. mesenteroides*	Unknown (Danisco)
PCD 215	*P. pentosaceus*	Unknown (Danisco)
PCD 240	*P. pentosaceus*	Unknown (Danisco)
PCK 18	*Leuconostoc* sp.	Maasai milk (Kenya)
PCK 37	Unidentified	Maasai milk (Kenya)
PCK 38	Unidentified	Maasai milk (Kenya)
PCK 73	Unidentified	Coffee fermentation (Ethiopia)
PCA 227	*Lactobacillus* sp.	Unknown
PCA 144	*L. fermentum*	Kasseri cheese
PCA 244	*L. reuteri*	Adult intestine
PCA 306	*L. plantarum*	Feta cheese
PCA 314	*L. plantarum*	Feta cheese
PCD 227	*L. plantarum*	Unknown (Danisco)
PCD 241	*L. plantarum*	Unknown (Danisco)
PCD 103	Unidentified	Unknown (Danisco)
PCK 40	*Lactobacillus* sp.	Maasai-milk (Kenya)
PCK 46	Unidentified	Maasai-milk (Kenya)
PCK 49	Unidentified	Maasai-milk (Kenya)
PCK 66	*Lactobacillus* sp.	Coffee fermentation (Ethiopia)
PCK 74	*Leuconostoc* sp.	Coffee fermentation (Ethiopia)
PCK 161	Unidentified	Unknown
PCK 88	*Lactobacillus* sp.	Coffee fermentation (Tanzania)
PCK 103	*Lactobacillus* sp.	Salgam (Turkey)
PCS 18	Unidentified	Cheese
PCS 20	Unidentified	Cheese
PCS 25	Unidentified	Cheese
PCB 12	*Bifidobacterium* sp.	Chicken
PCB 14	*Bifidobacterium* sp.	Chicken
PCB 26	*Bifidobacterium* sp.	Rumen
PCB 50	*Bifidobacterium* sp.	Mouse
PCB 51	*Bifidobacterium* sp.	Mouse
PCB 54	*Bifidobacterium* sp.	Rat
PCB 70	*Bifidobacterium* sp.	Rabbit

PCB 71	*Bifidobacterium* sp.	Rabbit
PCB 76	*Bifidobacterium* sp.	Rabbit
PCB 107	*Bifidobacterium* sp.	Calf
PCB 110	*Bifidobacterium* sp.	Calf
PCB 111	*Bifidobacterium* sp.	Calf
PCB 133	*Bifidobacterium* sp.	New-born
PCB 134	*Bifidobacterium* sp.	New-born
PCB 139	*Bifidobacterium* sp.	New-born
PCB 142	*Bifidobacterium* sp.	New-born
PCB 148	*Bifidobacterium* sp.	New-born
PCB 150	*Bifidobacterium* sp.	New-born
PCB 157	*Bifidobacterium* sp.	New-born
PCB 158	*Bifidobacterium* sp.	New-born
PCB 191	*Bifidobacterium* sp.	Piglets
PCD 232B	*B. longum*	Unknown (Danisco)
PCD 359B	*B.thermophilum*	Unknown (Danisco)
PCD 733B	*B.longum*	Unknown (Danisco)
PCD 735B	Unidentified	Unknown (Danisco)
PCD 880B	*B. adolescentis*	Unknown
PCD 889B	*B. longum*	Unknown (Danisco)

The strains, stored as frozen culture, were cultivated in agar plates, examined for colony morphology and subjected to microscopic observation. Only one colony from agar plate was sub-cultivated in appropriate broth medium, then streaked again on agar plates and, again, only one colony was taken out and cultivated in broth medium**,** in order to ensure the purity of the strain.

Cultivation of strains

The "stock culture" of the LAB strains were kept in skim milk at -80°C. At first, two subcultures were made from the stock culture to obtain a fresh, exponentially growing cell culture. Bifidobacteria were cultured in TPY (Tryptone, Peptone, Yeast, see table 11) broth (Scardovi, 1986; Modesto *et al.,* 2003) at 37°C for 24-48 hours in anaerobic conditions generated by using Anaerocult A (Merck, Darmstadt,

Germany), were cultivated in MRS (DeMan-Rogosa Sharpe) broth (Merck, Darmstadt, Germany) at 30°C or 37°C depending on the species; in anaerobic conditions as described above for 18-24 hours.

Tab. 11. Composition of TPY broth

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

The strains of *Campylobacter* were grown on Nutrient agar (Oxoid, Ltd., Basingstoke, Hampshire, England) containing 5% sheep blood at 42 °C under microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) generated by using CampyGen Atmosphere Generation System (Oxoid, Ltd., Basingstoke, Hampshire, England) in anaerobic jars for 24-48 hours. Thereafter, one typical colony of each strain of *Campylobacter* was transferred into NB (Nutrient broth) (Oxoid, Ltd., Basingstoke, Hampshire, England) supplemented with 5 % of Laked Horse Blood (Oxoid, Ltd., Basingstoke, Hampshire, England), kept under microaerophilic conditions for 48 hours at 42 °C and then used for the experiment.

Agar Spot Test using living cells

Preparation of the *Campylobacter* strains

Campylobacter strains were grown until the broth culture achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 24-48 hours). The turbidity of the actively growing culture was adjusted with sterile broth to obtain a turbidity optically

comparable to that of the 0.5 McFarland standard. This results in a suspension containing approximately 1 x 10^7 cfu/ml (Kuana et al., 2008; Andrews, 2001). To perform this step properly adequate light is needed to visually compare the inoculum tube and the McFarland standard against a card with a white background and contrasting black lines.

Work procedure

TPY agar or MRS agar (depending on the strain used) was poured in petri dishes until they solified. Then, 10 µl of LAB or bifidobacteria growth were spotted onto fresh lawns of the appropriate agar (TPY agar or MRS agar) and after 30 minutes, plates were incubated in anaerobic conditions for 24 hours at 37°C.

Subsequently, the plates were overlaid with 10 ml of NB (Nutrient Broth) (Oxoid) additionated with 0,7% agar to obtain a "Soft" agar, containing 100 µl of the *Campylobacter* cell suspension as described in previous section. Then, petri dishes were left to dry and incubated anaerobically at 37°C. After 24-48 hours were observed the inhibition zones. Each assay was performed in duplicate and 5 µl of acetic acid (1 M) was used as a positive control and sterile MRS or TPY broth at pH 6.5 were used as a negative control.

The Spot Agar test was performed also by using cell-free culture supernatants.

Agar Spot Test using cell-free culture supernatants

Preparation of cell-free culture supernatants

LAB and bifidobacteria strains grown overnight in 9 ml of appropriate media were harvested by centrifugation at 15.000 *g* at 4°C for 15 minutes, twice. Pellets were removed and supernatants were adjusted to pH 6.5 with 10 N NaOH (to exclude the effect of organic acids) to obtain the "NCSs" (Neutralised cell-free culture supernatants).

Work procedure

As in the spot agar test using living cells, a TPY agar or MRS agar (depending on the strain used) was put in petri dishes and left to solify. 10 µl of LAB cell-free culture supernatants prepared as described above were spotted onto fresh lawns of the appropriate agar (TPY agar or MRS agar) and after 30 minutes, the plates were incubated in anaerobic conditions for 24-48 hours at 37°C.

Subsequently, the plates were overlaid with 10 ml of nutrient broth 0.7% of agar, containing 100 µl of the *Campylobacter* cell suspension. The petri were incubated for 24 hours at 37°C in anaerobic conditions and the inhibition zones were observed. The experiment was duplicated. 5 µl of acetic acid (1 M) was used as a positive control and sterile MRS or TPY broth at pH 6.5 were used as a negative control.

Well Diffusion Assay

In the well diffusion assay, an actively growing broth culture of *Campylobacter* strain was adjusted with sterile broth to obtain a suspension of about 1 x 10^7 cfu/ml. Then, 500 µl of this cells suspension was added to 20 ml of Nutrient Agar (1,5 % agar) (Oxoid, Ltd., Basingstoke, Hampshire, England), poured into petri dishes, and allowed to solidify at room temperature. Wells (5 mm in diameter) were made on the solidified agar with a sterile pasteur or sterile metal cylinder, and were filled with 50 µl of NCSs from each strain tested. After 48 hours of incubation in microaerophilic conditions at 42°C, the inhibition zones were observed. Each assay was performed in duplicate, and also in this case 5 µl of acetic acid (1 M) was used as a positive control, sterile broth as a negative control.

This test was performed on PCA 227, PCA 236, PCA 263, PCA 275, PCK 73, PCK 18, PCK 103, PCD 733 B, PCD 103, PCB 133 and PCS 20 strains, as they had shown an antagonistic activity towards *C jejuni* in the previous spot agar test.

Chapter 2. Screening for survival in gastrointestinal tract and food processing conditions

The screening for survival in the gastrointestinal tract (low pH and presence of bile salts) and tolerance towards stress conditions prevailing in the food processing (heat treatment, osmotic stress and starvation) was performed on the 11 strains selected from previous antimicrobial activity tests.

Enumeration of viable cells

Viable cells were enumerated by plating diluted samples (peptonized water or saline solution) on MRS or TPY agar. Cultures were diluted 1:10 (1 part culture to 9 ml peptonized water or saline solution). Cultures were incubated for 18-24 hours (depending on strains) in anaerobic jars at 37°C to determine the population. The bacterial growth was expressed in colony forming units per milliliter (cfu/ml) and the survival percentage (% ± sd) of strains to different treatments was then calculated.

Buffers, reagents and media:
Man Rogosa Sharpe (MRS) broth medium was used to cultivate isolated lactic acid bacteria, as well as MRS agar (Merck, Darmstadt, Germany). TPY (Tryptone, Peptone, Yeast extract) medium was used for bifidobacteria and prepared in according to the indication of Scardovi (1986). The following buffers were used:
PBS buffer: K_2HPO_4 1.41 g/l; KH_2PO_4 0.26 g/l and sodium chloride 8.0 g/l. Peptone water: Peptone 10.0 g/l; sodium chloride 5.0 g/l; disodium hydrogen phosphate

dodecahydrate 9.0 g/l; potassium dihydrogen phosphate 1.5 g/l (pH 7.2). PBS (Phosphate Buffered Saline, Dulbecco A) buffer was obtained from Oxoid (Oxoid, Ltd., Basingstoke, Hampshire, England); peptone water was obtained from Merck; saline tablets were obtained from Oxoid. Solutions were prepared in 100 ml volume and autoclaved at 121 °C for 20 min and stored at room temperature until used.

Resistance to low pH

Berrada et al. (1991) reported the time from the entrance to the release from the human stomach to be 90 min. Thus, the strains selected to be used as probiotic bacteria should be able to tolerate acid for at least 90 min.

Growth, harvesting of cells and work protocol for the evaluation of the effect of pH on survival:

Lactobacilli and enterococci from stock culture at -80°C were once subcultured in 10 ml of MRS broth for 18 hours at 37 °C depending on species, in anaerobic conditions. The final growth was obtained after another subculture in 10 ml of MRS broth for 18 hours at 37°C. The bifidobacteria were subcultured twice in 10 ml of TPY broth for 24-48 hours at 37°C in anaerobic conditions.
Effect of pH on survival was determined following the procedure of Huang et al. (2007).
For all strains, the initial population was adjusted with sterile saline solution to approximately 10^9 cfu/ml. Then, 1 ml was taken as reference value (T0): the number of viable cells was determined by serial 10-fold dilution in peptone water 1 ml aliquots were inoculated evenly on MRS or TPY agar. The plates were incubated anaerobically at 37°C for 24-48 hours and the colony forming units estimated. The verification of the identity of the colonies was carried out through observation at microscope. The remaining 9 ml were centrifuged at 12.000 rpm for 10 minutes at

20°C and the supernatants were removed under aseptic conditions. 9 ml of sterile PBS buffer at pH 7.2 were added to pellets and the tubes were vortexed to obtain a full resuspension. Then, another centrifugation was carried out at 12.000 rpm for 10 minutes at 20°C and the supernatants were removed again. Finally, 9 ml of sterile PBS buffer at pH 2.5 were added and the tube vortexed again. Then, the tubes were incubated at 37°C, and after 30 minutes, 1,2 and 3 hours, 1 ml was taken and diluted serially in peptone water or saline solution (in case of Bifidobacteria), and finally plated with MRS or TPY agar. The plates were incubated for 24-48 hours at 37°C, in anaerobic atmosphere, for cfu determination. Sterile double distilled water (pH 6.4) served as a control. Each experiment was repeated four times.

Resistance to bile salts

In humans, after a meal, the bile salt concentration sharply increases in the duodenum up to about 15 mmol/L and then progressively decreases to 5 mmol/L. In the jejunum, the bile salt concentration is about 10 mmol/L, and, in the ileum, the concentration falls below 4 mmol/L because of active ileal absorption.

Growth, harvesting of cells and work protocol for the bile tolerance test:

The bile salt solutions were prepared using Oxgall (Ox-Bile LP0055, Oxoid) powder. The powder was rehydrated by preparing 10 g dry powder base in 90 ml distilled water. From this solution, final concentrations of 0,5, 1, 2 and 4% w/v were prepared in PBS buffer and pH was adjusted to 8.0 with NaOH 1 M. Oxgall solutions were sterilized through a 0.22 μm filter and fresh prepared just before use. PBS buffer, peptone water and media were prepared as previously described.
From stock culture at -80 °C the 11 strains previously selected were subcultured in 10 ml of MRS or TPY broth for 18-24 hours depending on species at 37°C in anaerobic

conditions. The final growth was obtained after another subculture in 10 ml of MRS or TPY broth for 18-24 hours depending on species at 37 °C.

The resistance to bile salt was assayed following the procedure of Huang and others (2007): for all strains, the initial population was adjusted with sterile saline solution to approximately 10^9 cfu/ml and one millilitre of culture was taken out from each tube immediately as reference value (T0). For the remaining 9 ml, the cells were centrifuged at 12.000 rpm for 10 minutes at 20°C. The supernatants were discarded, and the cell pellets were washed once with PBS buffer pH 7.2 and resuspend in it. The cells were centrifuged again at 12.000 rpm for 10 minutes at 20°C and the supernatants were removed. Nine milliliter of sterile PBS buffer at pH 8 containing 1 or 2% (w/v) Oxgall were added to the pellets and the tube vortexed again for full resuspension. The number of surviving cells was determined after the anaerobic incubation at 37°C at timed intervals (0,5, 1, 2 and 4 hours) by plating them on MRS or TPY agar medium after serial dilution in peptone water (for LAB and enterococci) or saline solution (for Bifidobacteria). Sterile double distilled water without oxgall (pH 6.4) was used as a control. CFUs were counted after 24-48 hours, depending on species. The verification of the identity of the colonies was performed through observation at microscope. Each experiment was repeated four times.

Heat stress

Lactic acid bacteria are extensively used in the dairy industry, consequently they have to face the various harsh conditions imposed by industrial processes. One of the most recurrent conditions could be the exposure to high temperature**.**

Lactic acid bacteria are used as starters for manufacture of cheese and some kinds of cheeses are traditionally fermented at a naturally decreasing temperature from about 55°C (cooking temperature of the curds) to 30 °C or below.

Growth, harvesting of cells and work protocol for resistance to heat stress:

The strains were grown in 10 ml of MRS or TPY broth twice from stock cultures until a final population of about 10^9 cfu/ml was obtained. Then, one milliliter was taken as reference value. The remaining part of the broth grown cultures were harvested (12.000 rpm for 10 minutes at 20°C) two times and resuspended in 10 ml of sterile PBS (pH 7.2). The last PBS was preheated at the required temperature (50°C and 55°C). Then, the cultures were transferred to water baths maintained at 50 and 55°C for 15, 30 and 60 minutes. At the end of the time samples were chilled on ice for 10 minutes, diluted and plated on MRS or TPY agar and incubated anaerobically at 37°C for 24-48 hours, in relation to the different species. Finally, CFUs were counted. Tests were performed four times.

Osmotic stress

In their various applications in the food and feed industry, lactic acid bacteria can be exposed to osmotic stress when important quantities of salt or sugar are added to the product.

Growth, harvesting of cells and work protocol for resistance to heat stress:

From frozen stock cultures, the strains were subcultured twice in 10 ml MRS or TPY broth. After 18-24 hours of incubation in anaerobic atmosphere at 37 °C (to afford about 10^9 cells per milliliter), 1 ml was pipetted out and immediately 10-fold diluted in peptone water to estimate the CFUs. The rest of the culture was harvested by centrifugation at 12.000 rpm for 10 min at 16^{o}C and the supernatants were removed; sterile PBS buffer at pH 7.2 was used for washing and suspending the cells. Then, the cells were harvested again at the same conditions and the pellets were resuspended in PBS buffer at pH 7.2, containing 6 % NaCl, vortexed and incubated under the previously described conditions. After 0, 30 and 60 minutes one milliliter was taken

out and serially diluted in peptone water (0.9% NaCl for bifidobacteria) and then plated in MRS or TPY agar. The plates were incubated for 24-48 h, depending on species, at 37^{o}C, for CFUs determination. Experiments were repeated three times.

Starvation stress

Nutrient starvation is one of the most frequent stresses for bacteria. Bacterial growth itself contributes to the nutrient exhaustion and the subsequent starvation for one or several compounds.

Growth, harvesting of cells and work protocol for resistance to starvation stress:

The stock cultures of the LAB strains were kept in skim milk at -80°C. First, two subcultures were made in 10 ml of MRS or TPY broth to obtain a fresh, exponentially growing cell cultures; the culture conditions were the same of the precedent protocols.
In order to standardize the assay, the initial population was approximately 10^{9} cfu/ml. From this solution 1 milliliter was taken as reference value. The rest was centrifuged (12.000 rpm, 10 min, 15^{o}C), the supernatants discharged and the pellets were washed with sterile PBS buffer (pH 7.2), twice. The suspensions were then incubated at 37°C in anaerobic conditions and after 6, 12 and 24 hours, 1 milliliter was taken out, serially diluted in sterile peptone or saline water for the determination of the CFUs, plated in the appropriate agar medium (MRS or TPY) and incubated for 24-48 hours at 37°C anaerobically. Experiments were repeated three times.

Chapter 3. Hemolytic activity

In the selection of strain for its putative probiotic characteristics its security is essential. The hemolysis is a common virulence factor among pathogens, it serves mainly to make iron available to the microbes and causes anaemia and oedema to the host.

In the ordinary laboratory practice the hemolytic activity of a specific strains is tested by streaking it on agar plates additioned with a sheep or human blood and the areas adjacent to the colony to be tested are put under observation. When Alpha hemolysis (α-hemolysis) is present the colony is surrounded by a zone of intact but discolored erythrocytes that have a green or brownish-green color. This appearance is generally due to the action of peroxide produced by the bacteria. This is sometimes called "partial hemolysis".

Beta hemolysis ((β-hemolysis), sometimes called "complete hemolysis", is a complete lysis of red cells in the media around and under the colonies: the area appears lightened and transparent. In the clear zone few or no intact erythrocytes are found. This reaction is best seen when the organism is growing under reduced oxygen concentration (the peroxide production is thereby decreased). β hemolysis is caused by one or more erythrocyte-lysing enzymes (hemolysins). If an organism does not induce hemolysis, it is said to display gamma hemolysis (γ-hemolysis), the agar under and around the colony remains unchanged (this is also called "non-hemolytic").

Work protocol for the assay of hemolytic activity

In the procedure for hemolytic activity of putative probiotic bacteria, the strains were once subcultured in MRS or TPY broth and incubated 18-24 hours at 37°C in anaerobic conditions. Then, fresh bacterial cell cultures were streaked on Columbia agar plates, containing 5 % of sheep blood. The plates were then incubated for 24 hours at 37 °C in anaerobic jars. As suggested by Maragkoudakis and collaborators (Maragkoudakis et al., 2009), the strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) were considered non hemolytic. The strains showing blood lysis zones around the colonies were classified as hemolytic (β-hemolysis). Experiments were performed in triplicate.

Chapter 4. Identification of strains

The identification was performed on the 11 strains that have shown antimicrobial activity against the tested pathogens with phenotypic methods (for the strains PCA 227, PCA 236, PCA 263, PCA 275, PCK 73, PCK 18, PCK 103, PCD 103, PCS 20) and molecular methods (for the above listed strains plus PCB133 and PCD 733B).

Phenotypic identification

Sugar fermentation reactions were performed using API 50 CH test strip and 50 CHL medium (bioMérieux, Marcy l'Etoile, France) which represent a standardized system for the identification of bacteria. API 50 CH strip consist of 50 microtubes used to study the fermentation of substrates belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids). During incubation, fermentation is revealed by a colour change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the medium. The first tube, which does not contain any active ingredient, is used as a negative control. API 20 STREP system was used to perform a phenotypic identification of the strain belonging to the genus *Enterococcus*. It is a standardized systems for the identification that uses enzymatic tests and a specific database. The strains are obtained according to the manufacturer's instructions and performed in duplicates. Profiles were determined with APILAB PLUS software program version 4.0. The tests are performed on PCA

227, PCA 236, PCA 263, PCA 275, PCK 73, PCK 18, PCK 103, PCD 103 and PCS 20 strains.

Molecular identification

From stock cultures (-80 °C), the strains were cultured in MRS or TPY broth in anaerobic conditions, 37 °C. After 18-24 hours (depending on the species), the strains were streaked into plates containing appropriate agar medium, and let grow cultivated left to grown in anaerobic conditions at 37 °C. The colonies formed on the media are picked with a sterile toothpick, and suspended in 50 µl of TE (10 mM Tris-HCl, 1mM EDTA; pH 8). The DNA extraction was conducted with the methodology of Matsuki *et al.* (2003). The bacterial suspension was heated at 95°C for 10 minutes, and 1 µl of the supernatant was subjected to PCR. All the TE suspension samples were stored at -20 °C.

Molecular identification at the species level of the two *Bifidobacterium* strains

Preliminary biochemical texts (fosfochetolase tests, sugar fermentation, PAGE pattern of soluble protein) and microscopic observations previously performed allowed to assign the two strains (PCB 133 and PCD 733B) to the *Bifidobacterium longum* species. In this work, the assignment of the two strains to the *B. longum* species was confirmed via PCR methods as described below.

Species-specific primers designed by Matsuki *et al.* (1999) are used (primer BiLON-1 and primer BiLON-2). PCR was carried out in a total volume of 25 µl of reaction mixture containing 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 2.5 mM of $MgCl_2$ (Applied Biosystems, Foster City, Ca), 200 µM each dNTP (Fermentas GmbH**,** St. Leon-Rot, Germany) 25 µM of BiLON-1 (5'-TTCCAGTTGATCGCATGGTC-3') (EurofinsMWG Synthesis GmbH, Ebersberg, Germany) and 25 µM of BiLON-2 (5'-GGGAAGCCGTATCTCTACGA-3') (Eurofins, MWG), 0.45 U of Taq DNA

polymerase (Fermentas) and 1 µl of template DNA. The PCR amplification program consisted of one cycle of 94 °C for 5 minutes, then 35 cycles of 94 °C for 20 seconds, 55 °C for 20 seconds, and 72 °C for 30 seconds, and finally one cycle of 72 °C for 5 minutes. Amplifications were carried out with a DNA thermocycler ((Biometra, Göttingen, Germany). The amplification products were then separated by electrophoresis in 1.5% (w/v) agarose gel and ethidium bromide (0.5 µg/ml) staining was performed to observe the presence of bands under UV transillumination (Bio-Rad) and photographed (Nikon, Tokyo, Japan). A positive control was performed by using DNA from a *B. longum* type strain from our collection and the negative control wqas performed by using water instead of DNA.

Molecular identification of the *Lactobacillus* spp. strains

The results of the API systems support that the PCA 236, PCA 263, PCA 275, PCS 20 and PCK 73 strains belong to the same species *L. plantarum*. To perform the molecular identification of these strains, a PCR with species-specific primers was done.

For species specific amplification of *Lb. Plantarum*, the primer pair *Lplan-vreg1-F/Lpla-vreg1-R* (Eurofins MWG) designed by Klocke *et al*. (2006), were used. PCR reactions were performed in a 25 µl mixture volume containing: 1x GeneAmp PCR Buffer II without $MgCl_2$ (Applied Biosystems), 2.5 mM $MgCl_2$, 100 ng of DNA template, 250 nM each primer, 0.25 mM (each) dNTP (Fermentas GmbH) and 0.625 U/µl AmpliTaq Gold DNA Polymerase (Applied Biosystems). PCR reactions were carried out in a TGradient Thermocycler (Biometra) according to the following amplification profile: a first cycle of incubation of 3 min at 95° C, then 30 cycles of amplification, including 30 sec at 94° C, 1 min at 57° C, and 90 sec at 72° C and a final cycle of 3 min at 72° C. Eight µl portions of the PCR products were electrophoresed in a 1.5% agarose gel and were subsequently visualized by UV illumination after ethidium bromide (EtBr) staining. The ladders used are GeneRuler

100 bp DNA Ladder Plus or GeneRuler 1 kb DNA Ladder Plus (Fermentas). Agarose gel pictures were taken shooting with a digital camera CoolPix 5400 (Nikon Corporation, Tokyo, Japan) equipped with special filters for EtBr and a compact camera hood (MicroBiotech, Bologna, Italy). Pictures were finally normalised with Nikon Editor Software 6.2 (Nikon).

Molecular identification of the *Enterococcus durans* strain

The results of the API systems support that the PCD 103 belong to the species *Enterococcus durans*. Identification was confirmed via PCR. The extraction of DNA from strain PCD 103 was carried out as previously described. The primers used **were** those designed by Knijff et al. (2001), precisely: DuHiF (5'-TTATGTCCCAGTATTGAAAAATCAA-3') and DuR (5'-TGAATCATATTGGTATGCAGTCCG-3'), both purchased from MWG. PCR was performed in 20 µl reaction mixture containing 2 µl 10 x PCR Buffer (Polymed, Florence, Italy), 1.25 µmol/l of each primer, 300 µmol/l of each dNTP, 6 mmol/l $MgCl_2$, 0.5 U of Taq DNA polymerase (Fermentas) and 2 µl of DNA, extracted as previously described. The amplification was carried out in a thermal cycler, programmed as follows: an initial denaturation step of 94 °C for 5 min, 30 cycles of 94 °C for 45 sec, 57 °C for 45 sec and 72 °C for 45 sec, followed by a final extension at 72 °C for 7 minutes. The amplification products were electrophoresed at 80 V on a 1.5% (wt./vol.) agarose gel stained with ethidium bromide and photographed.

Molecular identification of the *Lactobacillus pentosus* strain

For species-specific amplification of *Lactobacillus pentosus*, the primers pair *16S/Lpe* (EurofinsMWG) designed by Berthier and Ehlrich (1998) were used. PCR reactions were performed in a 25 µl mixture volume containing: 1x GeneAmp PCR

Buffer II without $MgCl_2$ (Applied Biosystems), 2.5 mM $MgCl_2$,100 ng of DNA template, 300 nM each primer, 0.25 mM (each) dNTP (Fermentas) and 0.625 U/µl AmpliTaq Gold DNA Polymerase (Applied Biosystems). PCR reactions were carried out in a TGradient Thermocycler (Biometra, Göttingen, Germany) according to the following amplification profile: a first cycle of incubation of 5 min at 95° C, then 30 cycles of amplification, including 1 min at 94° C, 1 min at 53° C, and 1 min at 72° C and a final cycle of 3 min at 72° C. Eight µl portions of the PCR products were electrophoresed in a 1.5% agarose gel and were visualized by UV illumination after ethidium bromide staining.

Molecular identification of the *Leuconostoc mesenteroides* strain

For species specific amplification of *Leuconostoc mesenteroides*, the primers pair *Lmes-F/Lmes-R* (Eurofins MWG) designed by Lee *et al*. (2000) were used. PCR reactions were performed in a 25 µl mixture volume containing GeneAmp PCR Buffer II without $MgCl_2$ (Applied Biosystems), 2.5 mM $MgCl_2$, 200 ng of DNA template, 500 nM each primer, 0.2 mM (each) dNTP (Fermentas) and 1.25 U/µl AmpliTaq Gold DNA Polymerase (Applied Biosystems). PCR reactions were carried out in a Veriti 96 well Fast Thermal Cycler (Applied Biosystems) according to the following amplification profile: a first cycle of incubation of 5 min at 95° C, then 30 cycles of amplification, including 1 min at 95° C, 1 min at 60° C, 2 min at 72° C and a final cycle of 10 min at 72° C. Eight µl portions of the PCR products were electrophoresed in agarose gel and visualized by UV illumination.

Molecular identification of the *Lactobacillus delbrueckii* strain

For species specific amplification of *Lactobacillus delbrueckii*, the primers pair *Ldelb-F/Ldelb-R* (Eurofins MWG) designed by Byun *et al*. (2004) were used. PCR reactions were performed in a 25 µl mixture volume containing 1 x HotStarTaq

Master Mix (Qiagen GmbH, Hilden, Germany) including 1.5 mM $MgCl_2$ and 0.2 mM (each) dNTP, 50 ng of DNA template and 100 nM of each primer. PCR reactions were carried out in a Veriti 96 well Fast Thermal Cycler (Applied Biosystems) according to the following amplification profile: a first cycle of incubation of 15 min at 95° C, then 40 cycles of amplification, including 15 sec at 95° C, 1 min at 62° C, and a final cycle of 3 min at 72° C. Eight μl portions of the PCR products were electrophoresed in a 1.5% agarose gel and were visualized by UV illumination after ethidium bromide staining.

Chapter 5. Antibiotic resistance profiles

Bacterial strains

All bacterial strains from genus *Lactobacillus* (strains PCA 227, PCA 236, PCA 263, PCA 275, and PCK 73) and *Leuconostoc* (PCK 18) were grown in MRS broth for 18 h on 37°C under aerobic conditions and all bacterial strains from genus *Bifidobacteria* (strains PCB 133 and PCD 733 B) were grown in TPY broth at 37 °C under anaerobic conditions. After 18 hours of incubation, 1 milliliter of the culture was taken out and transferred in 9 ml of fresh MRS or TPY broth, put in jars in anaerobic conditions at 37 °C for 18 hours.

Antibiotic solutions

The antibiotic resistance was assessed by cultivating bacterial strains in presence of antibiotics. The antibiotics used were: Tetracycline, Trimethoprim, Cefuroxime, Kanamycin, Chloramphenicol, Vancomycin, Ampycillin, Streptomycin (all from St. Louis, Mo., USA) and Erythromycin (from Fluka, Buchs SG, Switzerland). All antibiotic solutions were diluted in distilled water or DMSO or water with Ethanol (see table 12) to prepare stock solution and then additionally diluted with water to final concentration of 2, 4, 8, 16, 32, 64, 128 and 256 μg/ml for antibiotic resistance

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

Table 12. Solvents used for the antibiotic stock solutions.

Antibiotic	Solvent used
Tetracycline	distilled water
Trimethoprim	DMSO
Cefuroxime	distilled water
Kanamycin	distilled water
Chloramphenicol	distilled water with ethanol
Vancomycin	distilled water
Ampycillin	distilled water
Streptomycin	distilled water
Erythromycin	distilled water with ethanol

Antibiotic resistance assay

The assay was performed in 96 well plates by making serial dilution of antibiotics of concentration raging from 256 μg per ml to 2 μg per ml. In each well we added 20 μl of appropriate dilution of antibiotic, 160 μl of fresh MRS or TPY broth and 20 μl of overnight bacterial suspension previously diluted 1:9 in fresh MRS or TPY broth to obtain 10^6 CFU per ml. The number of tested bacteria was additionally determined by measuring the optical density (OD) at 620nm and through the use of a standard McFarland standards. The positive control in assay was a mixture of bacterial suspension (20 μl), broth (160 μl) and water or DMSO or water with ethanol (20 μl) without addition of antibiotic solution, and the negative control was a mixture of

bacterial suspension in water. Two additional controls were included; mixture of water, broth and antibiotic solution and broth only. The plates were then incubated for 24 hours in anaerobic conditions at 37 °C in order to determine the minimal inhibitory concentration. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic free control well and was measured by reading optical density at 620 nm. The MIC_{50} and the MIC_{90} were defined as the MIC that inhibited 50% and 90% of the tested microorganisms, respectively.

PART III

RESULTS

Chapter 1. Antimicrobial activity of LAB and bifidobacteria against *Campylobacter* strains

According to the results obtained with spot agar test with cell cultures, a high number of strains (15 strains out of the 60 tested) have shown an antimicrobial activity against the 3 strains of *C. jejuni*. Table 13 summarizes these results.

The strains which gave the best results were mainly belonging to the genera *Lactobacillus* (PCA 236, PCA 259, PCA 275, PCA 293, PCA 227, PCA 306) and *Bifidobacterium* (PCB 107, PCB 110, PCB 111, PCB 148, PCD 232B, PCD 359B, PCD 889B). Only the strains PCK 40 and PCB 71 have shown no activity with any of the three *Campylobacter* strains.

Considering that in the described spot agar test the antagonistic activities could be due to the production of organic acids from the bacteria, in order to assay whether this activity could be due to the formation of non-acidic products, spot agar tests using neutralised cell-free culture supernatants (NCSs) were performed. All the strains which had evidenced inhibition with at least one of the *Campylobacter* strains were employed in this experiments. When NCSs were spotted, the number of antagonistic strains against pathogens decreased dramatically, and only 11 strain showed an antimicrobial activity against at least one of the strain (listed in table 14).

Table 13. Inhibitory activity of cell cultures of strains, as determined with the agar spot test of Schillinger and Lücke (1989).

	Inhibitory activity*						
Strain	*Campylob. jejuni* CIP 70.2	*Campylob. jejuni* LMG 8842	*Campylob. jejuni* 221/05	Strain	*Campylob. jejuni* CIP 70.2	*Campylob. jejuni* LMG 8842	*Campylob. jejuni* 221/05
PCA 236	+	+	+	PCK 103	-	+	+
PCA 259	+	+	+	PCS 18	-	-	+
PCA 263	+	-	+	PCS 20	-	+	+
PCA 275	+	+	+	PCS 25	-	-	+
PCA 293	+	+	+	PCB 12	-	-	+
PCD 71	-	-	+	PCB 14	-	-	+
PCD 101	-	-	+	PCB 26	-	+	+
PCD 119	-	-	+	PCB 50	+	-	+
PCD 215	-	-	+	PCB 51	-	-	+
PCD 240	-	-	+	PCB 54	+	+	-
PCK 18	+	+	+	PCB 70	+	-	-
PCK 37	-	+	+	PCB 71	-	-	-
PCK 38	+	-	-	PCB 76	+	-	-
PCK 73	+	-	+	PCB 107	+	+	+
PCA 227	+	+	+	PCB 110	+	+	+
PCA 144	+	-	-	PCB 111	+	+	+
PCA 244	-	+	-	PCB 133	+	+	-
PCA 306	+	+	+	PCB 134	+	-	+
PCA 314	-	-	+	PCB 139	+	-	-
PCD 227	-	-	+	PCB 142	+	+	-
PCD 241	-	-	+	PCB 148	+	+	+
PCD 103	-	-	+	PCB 150	+	-	-
PCK 40	-	-	-	PCB 157	+	-	-
PCK 46	+	-	+	PCB 158	+	-	-
PCK 49	+	-	-	PCB 191	+	-	-
PCK 66	+	-	-	PCD 232B	+	+	+
PCK 74	+	-	+	PCD 359B	+	+	+
PCK 161	+	+	+	PCD 733B	-	+	+
PCK 88	-	-	+	PCD 735B	-	+	-

PCD 889B	+	+	+	PCD 880B	+	+	-

*+, clear inhibition zone ≥ 1 mm; -, no inhibition zone.

Table 14. Inhibitory spectrum of the pH neutralized cell-free supernatants of the LAB strains, as determined with the agar spot test of Schillinger and F K Lücke (1989).

	Inhibitory activity*						
Strain	*Campylob. jejuni* CIP 70.2	*Campylob. jejuni* LMG 8842	*Campylob. jejuni* 221/05	Strain	*Campylob. jejuni* CIP 70.2	*Campylob. jejuni* LMG 8842	*Campylob. jejuni* 221/05
PCA 227	-	-	+	PCD 103	-	-	+
PCA 236	-	-	+	PCK 18	-	-	+
PCA 263	-	-	+	PCK 73	+	-	+
PCA 275	-	-	+	PCK 103	-	+	-
PCS 20	-	-	+	PCB 133	+	-	-
PCD 733B	-	-	+				

*+, clear inhibition zone ≥ 1.0 mm; -, no inhibition zone.

These 11 strains were then submitted to well diffusion assay test, whose results are shows in table 15.

Table 15. Inhibitory activity of pH neutralized cell-free supernatants of the strains tested determined with the well diffusion assay

	Inhibitory activity*						
Strain	*Campylob. jejuni* CIP 70.2	*Campylob. jejuni* LMG 8842	*Campylob. jejuni* 221/05	Strain	*Campylob. jejuni* CIP 70.2	*Campylob. jejuni* LMG 8842	*Campylob. jejuni* 221/05
PCA 227	-	+	-	PCD 103	-	+	-
PCA 236	-	+	-	PCK 18	-	-	+
PCA 263	-	+	+	PCK 73	+	-	+
PCA 275	-	+	+	PCK 103	-	+	+
PCS 20	-	+	+	PCB 133	-	+	+

*+, clear inhibition zone ≥ 1.0 mm; -, no inhibition zone.

The results performed with the spot agar assay and with the well diffusion assays by using NCSs are not always in agreement; however, they clearly show that the antimicrobial activity depends on the indicator strain used and that the well diffusion assay is more sensible in the indication of the antimicrobial activity with respect to the spot agar assay. By using the well diffusion assay, 7 starins showed inhibitory activity against at least two of the three *Campylobacter* strains used.

Chapter 2. Screening for survival in gastrointestinal tract and food processing conditions

The gastrointestinal tract is the major location to affect the viability of LAB cells. The *in vitro* criteria used in this study for the selection of candidate probiotics are defined in the guidelines of FAO/WHO committee (Joint FAO/WHO Working Report, 2002). The *in vitro* screening of the survival of LAB in simulated GI tract conditions may have value in predicting the actual *in vivo* survival of a strain. According to Havennar and Husis (1992), the stabilities of LAB cells obtained from either *in vivo* or *in vitro* study are similar. Moreover, the type of the *in vivo* studies as well as the experimental parameters (i.e. mode of administration) can be designed most effectively by the outcome of the above *in vitro* test results. The tests were performed on the 11 strains which had shown marked antimicrobial activity against *Campylobacter* strains (i.e. those described in Table 13).

Resistance to pH

For acid tolerance study, PBS or human (or animal) gastric liquid could be used. In this study, the viable LAB counts of each strain were determined after 0, 30, 60, 120 and 180 minutes of incubation in PBS buffer (pH 2.5).

For statistical analysis, all plate count data were converted to log cfu ml^{-1} (% Survival). Survival rates of the strains varied during incubation (Table 16, 17 and fig. 10).

Results from Table 1 and 2 show that after 1 hour almost all strains have lost their viability. Only PCS 20 (7.89 log cfu ml^{-1}; 12.62% survival) and PCA 263 (6.04 log cfu ml^{-1}; 0.09% survival) strains retain their viability after 60 minutes of exposure.

Table 16. Survival[a] of strains tested at pH 2.5, value expressed as log cfu ml^{-1} at each sampling time

Strain	Viable counts of strains as log cfu ml^{-1} (SD)				
	0 min	**30 min**	**60 min**	**120 min**	**180 min**
PCA 227	9.08±0.82	6.78±1.50	5.17±1,29	4.60±2.94	0,70±0.58
PCA 236	9.45±1.00	7.70±0.96	5.1781.83	0.40±0.50	0.40±0.50
PCA 263	9.08±1.73	6.48±0.96	6.04±1.26	6.00±1.83	5.59±1.29
PCA 275	8.84±1.29	6.08±2.99	3.43±2.45	3.37±3.10	0.88±0.50
PCK 73	8.68±1.50	4.56±1.29	1.10±0.50	0.88±0.50	0.70±0.58
PCK 18	8.63±1.71	6.78±3.10	1.00±0.82	0.88±0.96	0.70±0.58
PCK 103	8.47±2.22	8.47±1.71	4.34±1.71	2.93±1.41	1.00±0.82
PCD 103	8.96±1.26	7.50±0.96	1.10±0.50	1.00±0.00	0.88±0.50
PCS 20	8.79±2.16	8.69±1.29	7.89±0.82	5.55±4.65	5.39±2.38
PCB 133	8.43±2.36	4.37±2.65	3.35±2.75	1.10±0.50	0.88±0.50
PCD 733B	9.90±4.11	3.08±1.29	2.95±1.41	1.00±0.00	1.00±0.82

[a] Each value in the table represents the mean value ± standard deviation (SD) from four trials.

Table 17. Survival of strain tested at pH 2.5, value expressed in % Survival.

Strain	Survival %				
	0 min	**30 min**	**60 min**	**120 min**	**180 min**
PCA 227	100%	0.51%	0.01%	0.00%	0.00%
PCA 236	100%	1.77%	0.01%	0.00%	0.00%
PCA 263	100%	0.25%	0.09%	0.08%	0.03%
PCA 275	100%	0.17%	0.00%	0.00%	0.00%
PCK 73	100%	0.01%	0.00%	0.00%	0.00%
PCK 18	100%	1.42%	0.00%	0.00%	0.00%
PCK 103	100%	98.48%	0.01%	0.00%	0.00%
PCD 103	100%	3.44%	0.00%	0.00%	0.00%

PCS 20	100%	81.15%	12.62%	0.06%	0.04%
PCB 133	100%	0.01%	0.00%	0.00%	0.00%
PCD 733B	100%	0.00%	0.00%	0.00%	0.00%

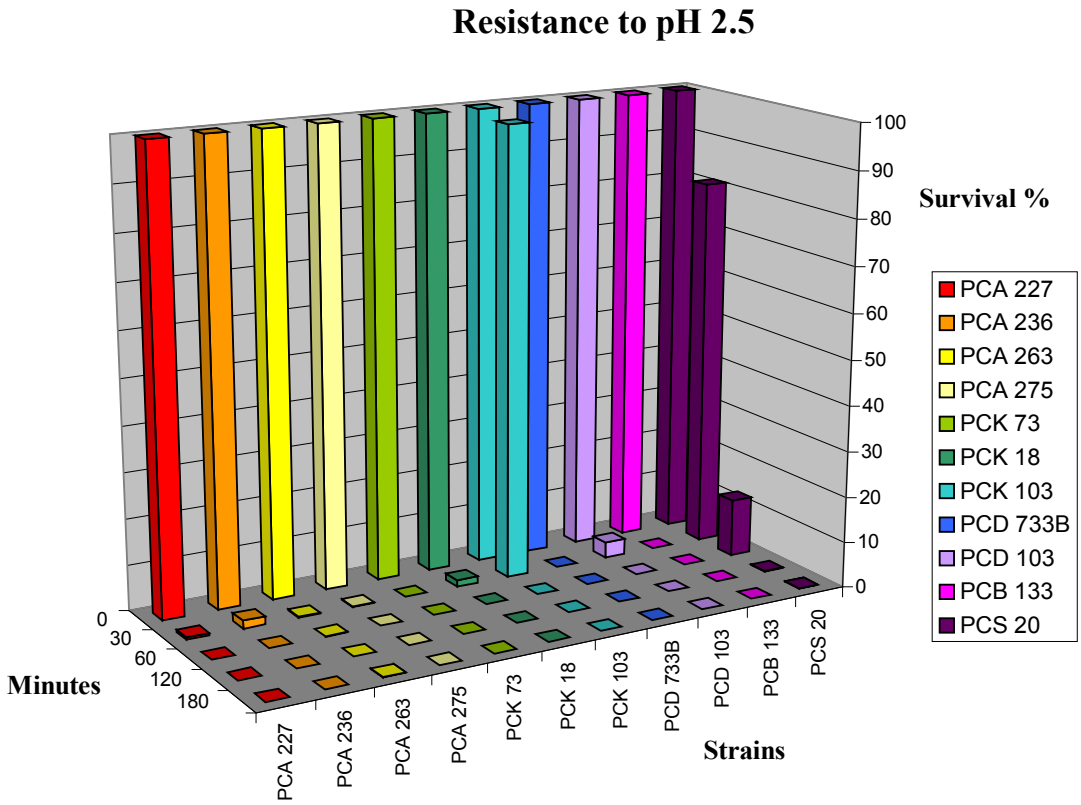


Fig 10. Resistance to pH 2.5

PCK 103 strain shows optimal resistance after 30 minutes (8.47 log cfu ml^{-1}; 98.48% survival), but its viability rapidly decreases after this time. After 3 hours of exposure to pH 2.5, viable counts were determined only for PCS 20 (5.39 log cfu ml^{-1}; 0.04% survival) and PCA 263 (5.59log cfu ml^{-1}; 0.03% survival).

Resistance to bile salts

The bile in human and animal intestine is also an important factor which affects the microrganism viability. Although the composition of human bile juice is not exactly the same as that of the oxgall solution, most studies use oxgall as one substitute for human and animal bile because of their similarity (Brashears et al., 2003).

Table 18. Effect[a] of Ox-gall 1% and 2% (w/v) on the growth of the 11 strains tested. Results are expressed as log cfu ml^{-1} (SD)

Strain	Ox-gall 1%			Ox-gall 2%		
	0 min	**60 min**	**240 min**	**0 min**	**60 min**	**240 min**
PCA 227	8.66±1.71	8.59±0.96	8.59±2.08	8.64±1.29	8.61±0.58	8.60±1.73
PCA 236	8.99±1.71	8.95±1.89	8.94±0.82	8.89±0.96	8.81±1.26	8.81±1.63
PCA 263	8.42±1.00	8.36±0.82	8.32±0.96	8.43±0.96	8.40±0.96	8.35±0.96
PCA 275	8.65±0.82	8.53±0.82	8.60±0.82	8.69±0.58	8.39±0.96	8.55±1.26
PCK 73	8.20±0.96	8.00±0.82	7.80±0.96	8.13±0.96	8.12±1.29	7.80±0.50
PCK 18	8.37±0.96	8.25±0.82	8.13±0.50	8.48±1.26	8.04±0.96	8.40±0.82
PCK 103	8.88±0.96	8.68±0.96	8.25±0.50	9.12±0.50	8.69±1.00	8.14±0.96
PCD 103	8.45±0.82	8.38±0.96	8.28±0.82	8.56±1.29	8.35±0.96	8.28±0.82
PCS 20	8.79±0.50	8.76±1.29	8.75±1.71	9.08±0.96	8.96±1.26	8.79±1.00
PCB 133	8.72±0.82	8.62±0.50	7.99±0.96	8.90±1.71	8.63±0.50	8.30±1.71
PCD 733B	8.01±2.87	7.58±0.96	6.51±0.50	8.14±0.58	7.22±1.50	6.44±0.50

[a] Each value in the table represents the mean value ± standard deviation (SD) from four trials.

Table 19. Survival of strains tested after exposure to Ox-gall 1% and 2%, value expressed in % Survival.

Strain	Ox-gall 1%			Ox-gall 2%		
	0 min	**60 min**	**240 min**	**0 min**	**60 min**	**240 min**
PCA 227	100%	85.85%	85.02%	100%	91.62%	90.71%
PCA 236	100%	90.33%	89.57%	100%	83.28%	83.32%
PCA 263	100%	86.79%	78.30%	100%	94.39%	83.18%
PCA 275	100%	75.56%	88.89%	100%	51.03%	72.68%
PCK 73	100%	64.43%	40.03%	100%	96.85%	47.31%
PCK 18	100%	75.08%	57.37%	100%	36.40%	82.50%
PCK 103	100%	62.62%	23.28%	100%	37.09%	10.52%
PCD 103	100%	86.61%	67.86%	100%	60.96%	52.05%

PCS 20	100%	92.37	90.36%	100%	77.04%	51.36%
PCB 133	100%	78.77%	18.40%	100%	52.65%	24.61%
PCD 733B	100%	36.92%	3.18%	100%	12.18%	2.00%

Probiotic strains were also examined for their ability to survive after exposure of 1% and 2% (w/v) oxgall solutions. CFUs were determined at time 0 and after 1 and 4 hours. Results were summarised in table 18, 19 and figures 11 and 12.

At Ox-gall concentration of 1% (w/v) more than 50% of the initial population survive after 1 hour of exposure, except for PCD 733B (7.58±0.96 log cfu ml^{-1}; 36.92% survival). At the same concentration of bile salt (1%), but after 4 hours, 7 of the eleven strains retain their viability of more than 50% of the initial population: PCA 227 (8.59±2.08 log cfu ml^{-1}; 85.85% survival), PCA 236 (8.94±0.82 log cfu ml^{-1}; 89.57% survival), PCA 263 (8.32±0.96 log cfu ml^{-1}; 78.30% survival), PCA 275 (8.53±0.82 log cfu ml^{-1}; 88.89% survival), PCK 18 (8.13±0.50 log cfu ml^{-1}; 57.37% survival), PCD 103 (8.28±0.82 log cfu ml^{-1}; 67.86% survival) and PCS 20 (8.75±1.71 log cfu ml^{-1}; 90.36% survival). Only PCD 733B strain shows lower resistance (6.51±0.50 log cfu ml^{-1}; 3.18% survival). This strains also rapidly lost its viability after 1 hour of exposure at 2% Ox-gall (7.22±1.50 log cfu ml^{-1}; 12.18% survival). The other strains retain their viability even after 4 hour at 2% Ox-gall, but PCB 133 (8.30±1.71 log cfu ml^{-1}; 24.61% survival) shows moderate resistance as well as PCK 103 (8.14±0.96 log cfu ml^{-1}; 10.52% survival) strain. Most strains show to resist better in 1% solution of bile salt than in the more concentrated one (PCA 236, PCA 273, PCK 103, PCD 103, PCS 20, PCB 133 and PCD 733B), but other strains show an opposite behaviour (PCA 263, PCK 73), probably due to intrinsic characteristics of the strain.

PCA 275 strain shows susceptibility to Ox-gall 1% (8.59±0.58 log cfu ml^{-1}; 75.56% survival) and 2% (8.39±0.96 log cfu ml^{-1}; 51.03% survival) concentrations after 1 hour of exposure, but this susceptibility decreases after 4 hours at both concentrations of the bile salts (8.53±0.82 log cfu ml^{-1}; 88.89% survival with Ox-gall 1% treatment

and 8.55±1.26 log cfu ml^{-1}; 72.68% survival with Ox-gall 2% treatment). This behaviour is probably the consequence of an adaptation of the strain to stress, as already evidenced in the literature by Anukam and Koyama (2007). Similar results are found for PCK 18 strain.

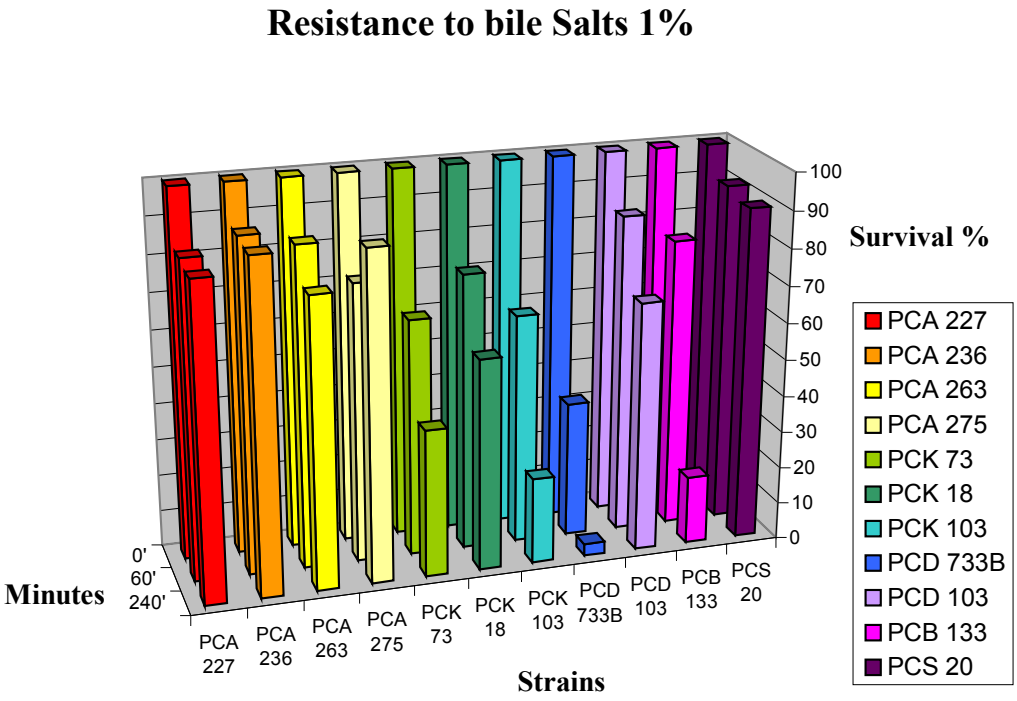


Fig. 11. Resistance to bile salts 1%

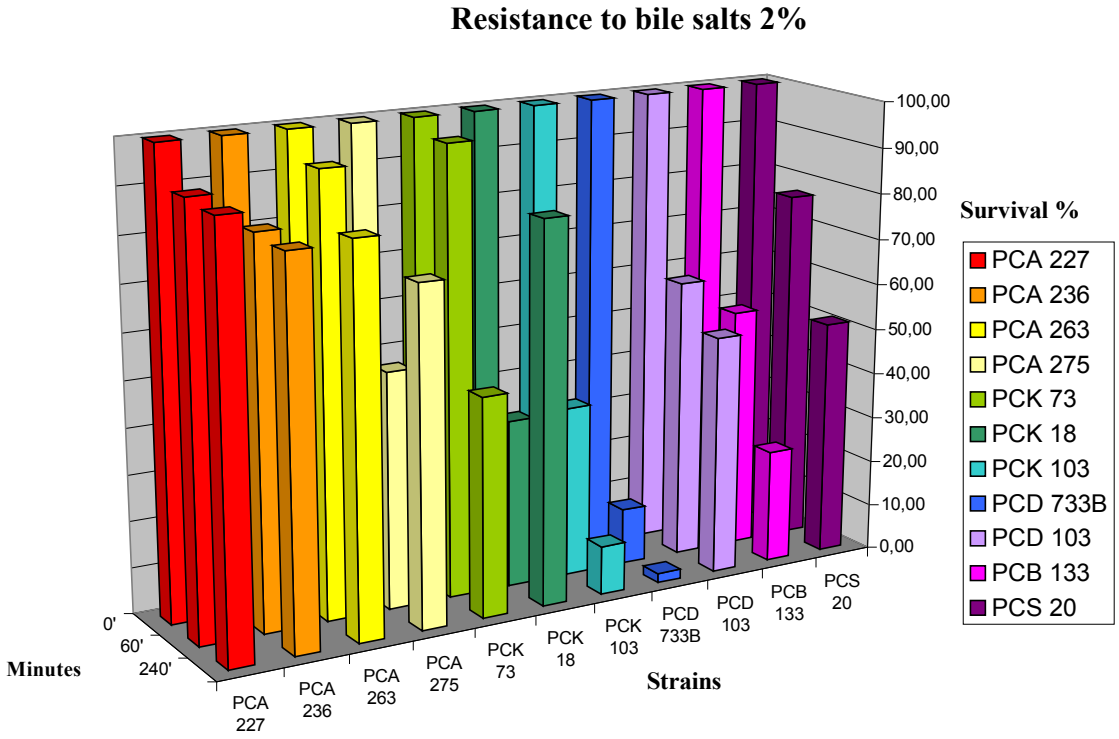


Fig. 12. Resistance to bile salts 2 %

Resistance to temperature

Thermophilic and thermotolerant organisms have an advantage since they survive higher temperature during processing and storage. They have a better chance of remaining viable during the process required for prolonged storage and they lead to a distinctly more effective product. In addition, thermophilic LAB are well-known for their biotechnological importance in the food industry.

In this work, the eleven strains were tested for their capacity to resist to 50 °C and 55 °C. Table 20 and 21 and figures 13 and 14 shows the effect of the heat treatment on the strains tested.

Table 20. Effect[a] of temperature (50°C and 55°C) on the growth of the 11 strain tested. Results are expressed as log cfu ml^{-1} (SD)

Strain	50°C				55°C			
	0 min	**15 min**	**30 min**	**60 min**	**0 min**	**15 min**	**30 min**	**60 min**

PCA 227	8.51±0.82	8.49±2.87	8.46±0.96	8.46±1.29	8.51±2.65	8.46±1.29	8.46±1.83	8.45±1.83
PCA 236	9.18±1.83	9.16±1.41	9.14±0.82	9.08±0.96	9.11±0.58	9.04±1.26	9.03±1.26	9.03±0.82
PCA 263	8.30±2.63	8.27±0.82	8.25±0.82	8.07±1.26	8.34±0.82	8.26±1.26	8.21±1.41	8.08±0.96
PCA 275	8.81±1.29	8.74±1.29	8.72±1.29	8.71±0.58	8.83±0.58	8.77±1.73	8.70±0.96	8.69±4.35
PCK 73	8.72±1.41	8.63±0.82	8.60±1.71	8.59±1.71	8.70±1.83	8.59±0.96	8.59±1.26	8.59±1.89
PCK 18	8.36±0.82	8.34±0.50	8.07±2.50	8.02±0.50	8.31±1.50	8.27±1.41	8.01±2.06	8.00±0.82
PCK 103	8.47±0.96	8.43±1.29	8.41±2.65	8.15±2.63	8.48±0.96	8.41±2.16	8.29±1.50	8.09±3.11
PCD 103	8.74±1.29	8.61±1.41	8.60±4.99	8.53±1.71	8.74±1.29	8.60±1.73	8.60±0.96	8.51±1.83
PCS 20	9.10±0.96	9.08±0.96	9.07±1.29	8.17±0.50	9.07±0.96	9.03±1.41	9.02±1.89	8.11±1.89
PCB 133	8.22±0.96	8.08±2.16	8.04±1.89	7.74±1.50	8.22±1.26	8.08±1.29	8.00±2.22	7.76±0.82
PCD 733B	8.72±1.29	8.71±0.82	8.71±1.41	8.70±1.50	8.72±2.16	8.70±1.71	8.69±3.56	8.67±0.58

[a] Each value in the table represents the mean value ± standard deviation (SD) from four trials.

Table 21. Survival of strains tested after heat treatment (50 °C and 55 °C), value expressed in % Survival.

Strain	**50°C**				**55°C**			
	0 min	**15 min**	**30 min**	**60 min**	**0 min**	**15 min**	**30 min**	**60 min**
PCA 227	100%	95.43%	88.93%	88.39%	100%	90.08%	89.30%	87.29%
PCA 236	100%	94.70%	92.05%	79.64%	100%	85.80%	83.85%	83.27%
PCA 263	100%	93.88%	89.39%	58.05%	100%	83.53%	74.19%	55.18%
PCA 275	100%	85.70%	80.89%	80.57%	100%	87.68%	74.57%	73.53%
PCK 73	100%	81.99%	76.77%	74.38%	100%	78.72%	78.82%	78.52%
PCK 18	100%	96.15%	52.09%	46.15%	100%	91.78%	50.67%	48.59%
PCK 103	100%	89.84%	85.81%	47.94%	100%	84.70%	65.10%	40.86%
PCD 103	100%	73.70%	71.66%	62.28%	100%	73.37.%	72.77%	59.14%
PCS 20	100%	94.50%	93.12%	11.59%	100%	92.51%	90.58%	10.92%
PCB 133	100%	73.44%	66.62%	33.23%	100%	73.14%	60.24%	34.60%
PCD 733B	100%	96.87%	96.49%	94.83%	100%	95.00%	94.29%	89.05%

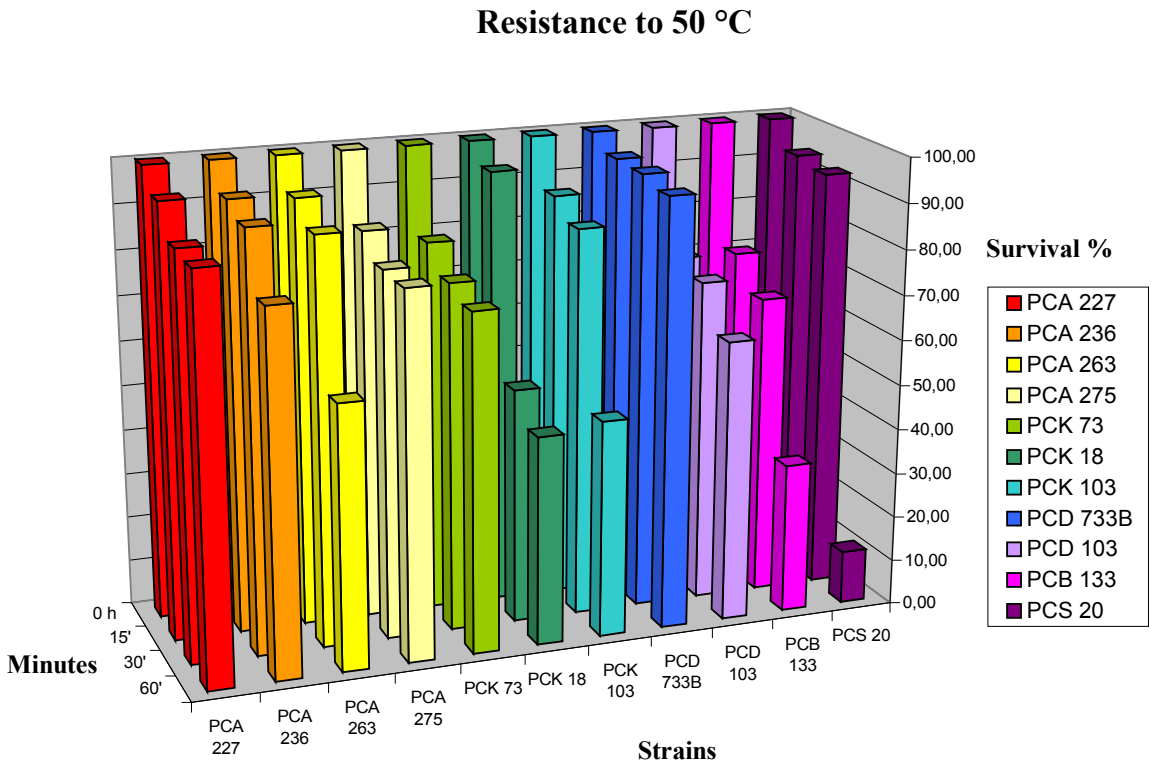


Fig. 13. Resistance of the strains tested to 50 °C

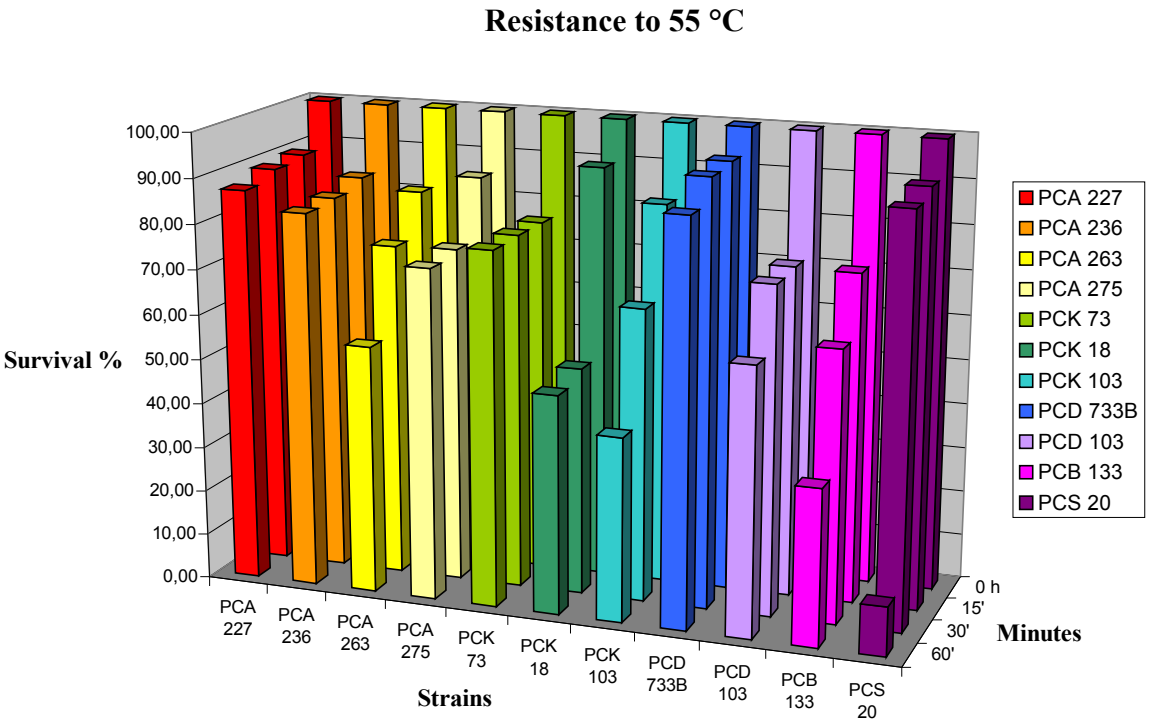


Fig. 14. Resistance to 55 °C

Tables 18 and 19 show that all strains are relatively resistants to both 50 °C and 55 °C heat treatments, even after 1 hour of exposure. Only PCS 20 was sensitive after 60 minutes at 50 °C (8.17±0.50 log cfu ml^{-1}; 11.59% survival) and 55 °C (8.11±1.89 log cfu ml^{-1}; 10.92% survival). PCB 133 strains shown moderate tolerance to 50 °C (7.74±1.50 log cfu ml^{-1}; 33.23% survival) and 55 °C (7.76±0.82 log cfu ml^{-1}; 34.60% survival) after 1 hour of heat stress, as well as PCK 103 (8.15±2.63 log cfu ml^{-1}; 47.94% survival at 50° and 8.09±3.11 log cfu ml^{-1}; 40.86% survival at 55 °C). In general, all strains show a better tolerance to the lower temperature heat treatment (50 °C) than to the higher one (55 °C).

Resistance to osmotic stress

In the different applications in the food and feed industry, lactic acid bacteria and bifidobacteria can be exposed to osmotic stress when important quantities of salt or sugar are added to the product. The effects of osmotic stress caused by high salt concentration on the viability of the selected strains is studied. Table 23 and figure 15 illustrate the percentage of survival of strains after 30 minutes and 60 minutes of exposure to NaCl 6% solution.

Table 22. Survival[a] of strains after exposure to NaCl 6%

Strain	Viable counts of strains as log cfu ml^{-1} (SD)		
	Time		
	0 min	**30 min**	**60 min**
PCA 227	8.83±1.00	8.81±0.58	8.79±0.58
PCA 236	8.90±1.73	8.89±0.58	8.83±1.00
PCA 263	8.41±3.21	8.21±1.53	7.76±0.58
PCA 275	8.75±0.58	8.72±1.53	8.68±0.58
PCK 73	8.15±2.65	8.00±1.15	7.99±1.00
PCK 18	8.35±3.21	8.35±1.00	8.32±2.65

PCK 103	8.93±0.58	8.90±1.53	8.79±2.08
PCD 103	8.71±2.65	8.49±2.65	8.63±2.52
PCS 20	9.53±1.53	9.43±1.15	9.27±1.53
PCB 133	8.45±1.00	8.40±1.00	8.34±1.00
PCD 733B	8.79±0.58	8.77±1.53	8.73±1.53

[a] Each value in the table represents the mean value ± standard deviation (SD) from three trials.

After 60 minute exposure, all the strains are relatively resistants: except for PCA 263 strain (7.76±0.58 log cfu ml^{-1}; 22.43% survival), all the strains retain their viability of more than 50% of the initial population, showing an optimal capacity to resist stress.

Table 23. Survival (%) of strains tested after a NaCl 6% treatment.

Strain	Survival %		
	0 min	**30 min**	**60 min**
PCA 227	100%	94.12%	91.67%
PCA 236	100%	97.08%	85.00%
PCA 263	100%	62.84%	22.43%
PCA 275	100%	94.61%	86.83%
PCK 73	100%	70.66%	69.01%
PCK 18	100%	98.96%	91.86%
PCK 103	100%	93.77%	71.60%
PCD 103	100%	83.30%	67.84%
PCS 20	100%	78.64%	55.02%
PCB 133	100%	90.00%	78.57%
PCD 733B	100%	96.74%	87.50%

The best results are done by PCA 227 (8.79±0.58 log cfu ml^{-1}; 91.67% survival), PCK 18 (8.32±2.65 log cfu ml^{-1}; 91.86% survival), PCA 236 (8.83±1.00 log cfu ml^{-1}; 85.00% survival), PCA 275 (8.68±0.58 log cfu ml^{-1}; 86.83% survival) and PCD 733B (8.73±1.53 log cfu ml^{-1}; 87.50% survival). For all strains, the viable cell counts decrease as the time of exposure to the solution NaCl 6% increases.

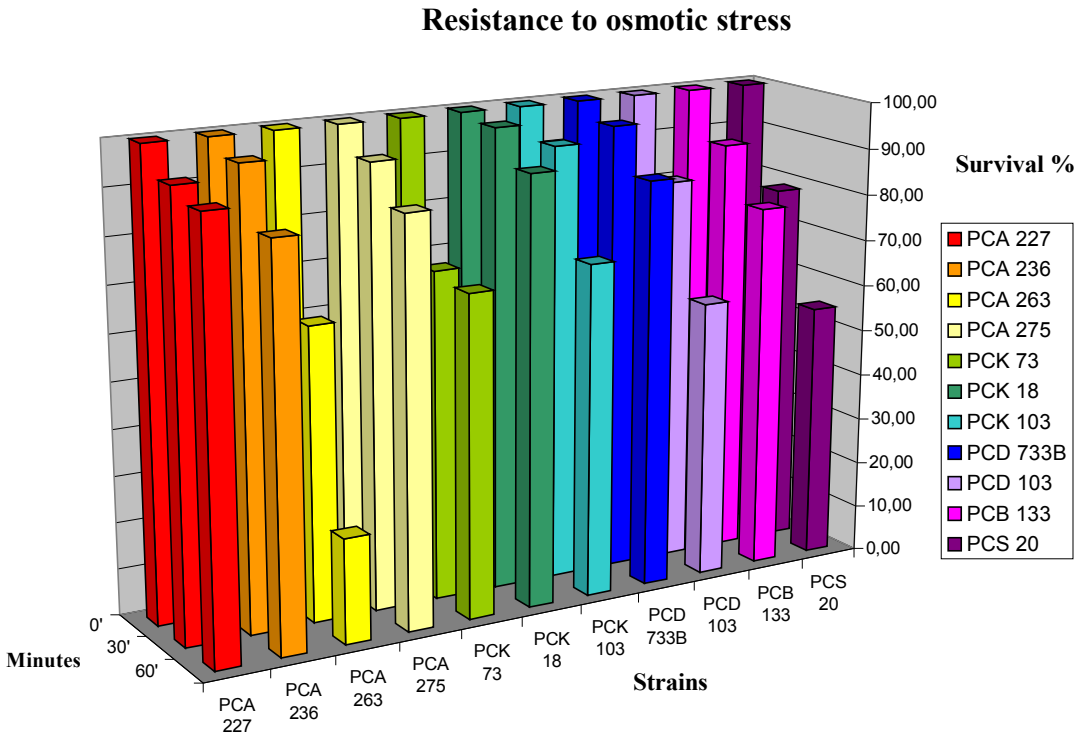


Fig. 15. Resistance to NaCl 6%.

Resistance to starvation stress

Stationary phase due to nutrient limitation corresponds to the usual conditions that microorganisms encounter in their natural environment, but also in particular condition, like the industrial transformation. In this study, the eleven strains were

tested for their survival after 6, 12 and 24 hours of starvation stress. Results are summarized in table 24 and 25.

Table 24. Survival[a] of strains tested after exposition to starvation stress

Strain	Viable counts of strains as log cfu ml^{-1} (SD)			
	Time (hours)			
	0 hours	**6 hours**	**12 hours**	**24 hours**
PCA 227	8.85±1.53	8.44±1.53	8.40±0.58	8.08±1.00
PCA 236	8.80±0.58	8.78±0.58	8.78±2.65	8.74±1.15
PCA 263	9.24±0.58	8.43±1.00	8.20±1.00	7.75±1.00
PCA 275	8.92±1.00	8.81±0.58	8.81±0.58	8.64±1.00
PCK 73	9.33±1.15	9.26±1.53	9.12±1.00	9.10±2.00
PCK 18	8.62±2.52	8.60±2.08	8.47±1.53	8.26±1.15
PCK 103	9.20±0.58	9.17±1.15	9.12±2.52	9.11±1.53
PCD 103	8.54±1.00	8.51±1.53	8.51±3.46	8.50±2.08
PCS 20	9.29±2.08	9.07±2.65	9.21±2.08	9.24±3.79
PCB 133	8.39±1.15	8.30±1.00	8.19±1.15	8.10±1.15
PCD 733B	9.08±1.00	8.56±1.53	8.46±1.00	8.44±0.58

[a] Each value in the table represents the mean value ± standard deviation (SD) from three trials.

Table 25. Survival (%) of strains after starvation stress.

Strain	**Survival %**			
	0 hours	**6 hours**	**12 hours**	**24 hours**
PCA 227	100%	39.15%	35.85%	16.98%
PCA 236	100%	95.79%	94.74%	87.37%
PCA 263	100%	15.40%	9.16%	3.21%
PCA 275	100%	77.91%	77.51%	53.01%
PCK 73	100%	84.29%	61.12%	59.25%
PCK 18	100%	94.99%	69.71%	43.72%

PCK 103	100%	92.68%	82.64%	81.17%
PCD 103	100%	94.61%	94.51	92.00%
PCS 20	100%	60.41%	83.11%	88.40%
PCB 133	100%	81.08%	63.51%	51.35%
PCD 733B	100%	30.56%	24.17%	23.06%

The most resistant strain is PCD 103, showing 92% of survival even after 24 hour of stress (8.50±2.08 log cfu ml^{-1}). PCA 236, PCK 103 and PCS 20 strains show an optimal resistance: their survival after the maximum time of stress are more than 80% of the initial population. After 24 hours, PCA 275 (8.64±1.00 log cfu ml^{-1}; 53.01% survival), PCK 73 (9.10±2.00 log cfu ml^{-1}; 59.25% survival), PCK 18 (8.26±1.15 log cfu ml^{-1}; 43.72% survival), PCB 133 (8.10±1.15 log cfu ml^{-1}; 51.35% survival) shows good resistance, while PCA 227 (8.08±1.00 log cfu ml^{-1}; 16.98% survival) and PCD 733B (8.44±0.58 log cfu ml^{-1}; 23.06% survival) have moderate capacity to resist, only PCA 263 (8.43±1.00 log cfu ml^{-1}; 15.40% survival) is already sensitive after 6 hours of treatment: its survival percentage after 24 hours is very low (3.21%).

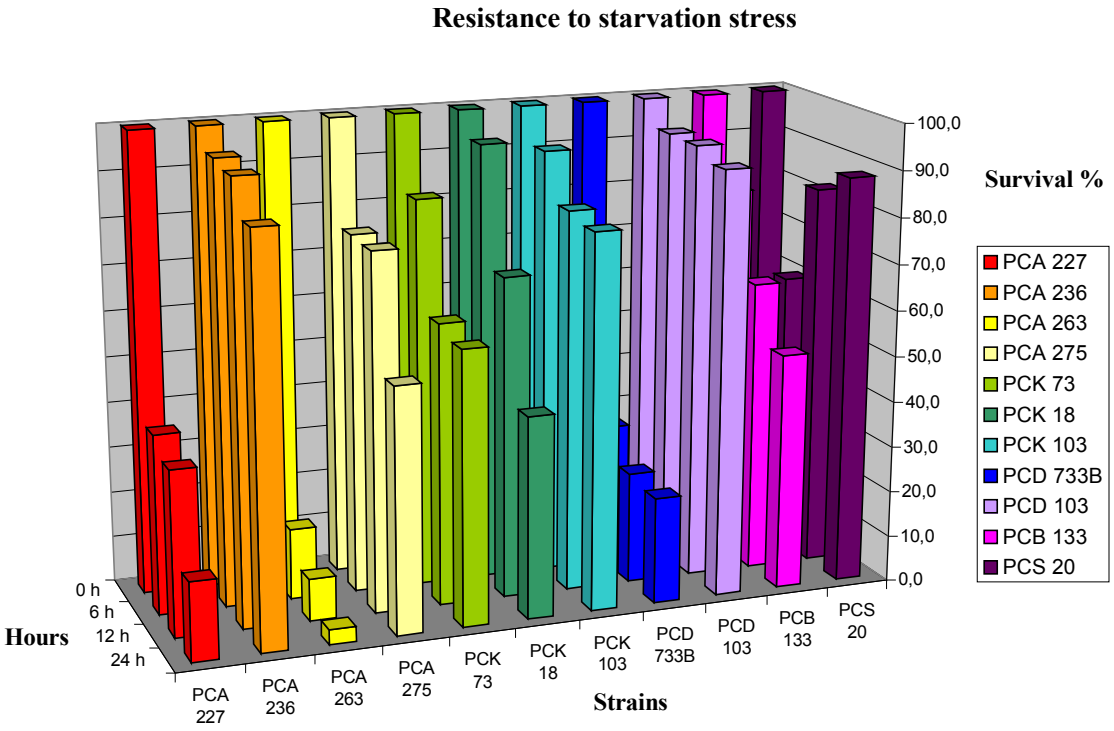


Fig. 16. Resistance to starvation stress.

Chapter 3. Hemolytic activity

The absence of hemolytic activity is essential for the selection of strains possessing putative probiotic characteristics. None of the tested strains exhibited β-hemolytic activities after 24 hours. Fig. 17 and 18 shows some results.

Fig. 17. PCA 275 strain in Columbia agar blood.. after 24 hours of incubation.

Fig. 18. PCS 20 strain (left) in Columbia agar blood after 24 hours of incubation.

Chapter 4. Identification of strains

Phenotypic identification

The biochemical identification of the 9 LAB which had shown interesting inhibitory activity towards *Campylobacter* spp. was performed with an API 50 CHL kit and API20 STREP. The identification results given by APILAB Plus software version 4.0 database are shown in Table 26:

Table 26. Identification of strains

Strain	Identification system	Strain	%ID (percentage of identification)
PCA 227	API 50 CHL	*Lactobacillus pentosus*	99.9%
PCA 236	API 50 CHL	*Lactobacillus plantarum*	99.3%
PCA 263	API 50 CHL	*Lactobacillus plantarum*	99.9%
PCA 275	API 50 CHL	*Lactobacillus plantarum*	91.1%
PCK 73	API 50 CHL	*Lactobacillus plantarum*	99.9%
PCK 18	API 50 CHL	*Leuconostoc mesenteroides*	85.5%
PCK 103	API 50 CHL	*Lactobacillus delbrueckii* ssp *delbrueckii*	95.2%
PCD 103	API 20 STREP	*Enterococcus durans*	91.9%
PCS 20	API 50 CHL	*Lactobacillus plantarum*	98.3%

For all strains, identification levels from good to excellent were obtained.

Molecular identification

To confirm biochemical identifications, species-specific PCR was performed on the DNA of all strains, as described in the section Matherial and methods. PCA 236, PCA 263, PCA 275, PCS 20 and PCK 73 strains were identified as *Lactobacillus*

plantarum. The DNA of *Lactobacillus plantarum* DSMZ 20174 strain was used as a positive control (Fig. 19).

PCD 103 strain was identified as *Enterococcus durans* as shows in Fig 20. Positive control used was DSMZ 20633 strain. Regarding PCB 133 and PCD 733B, biochemical and phenotypic characterization previously performed had allowed to identify them as *Bifidobacterium longum* strains. The identification was confirmed via species-specific PCR (Fig. 21). In the negative controls of all PCR analyses, sterile water was used in substitution of DNA.

PCA 227 strain was identified as *Lactobacillus pentosus* (Fig. 22), PCK 18 as *Leuconostoc mesenteroides* (fig. 23) and PCK 103 as *Lactobacillus delbrueckii* (fig. 24).

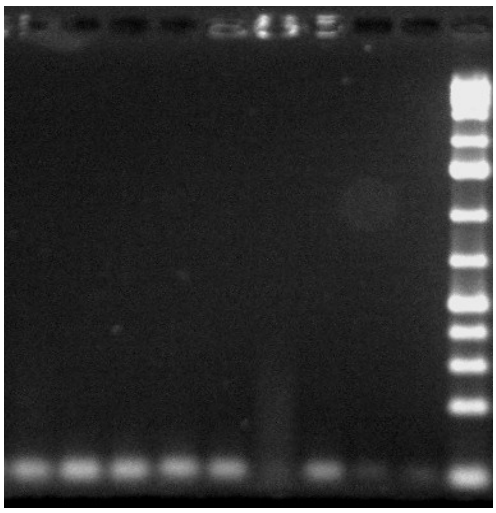


Fig.19. PCR electrophoretogram of *Lactobacillus plantarum* strains. 75 bp PCR products obtained for 7 *Lactobacillus* species and 1 *Leuconostoc* species with *Lactobacillus plantarum* specific primers. Lane 1, PCA227; lane 2, PCA236; lane 3, PCA263; lane 4, PCA275; lane 5, PCS20; lane 6, PCK18; lane 7, PCK73; lane 8, PCK103; lane 9, negative control (PCR performed with primers pair Lplan-vreg1-F/Lpla-vreg1-R and *E. coli*).

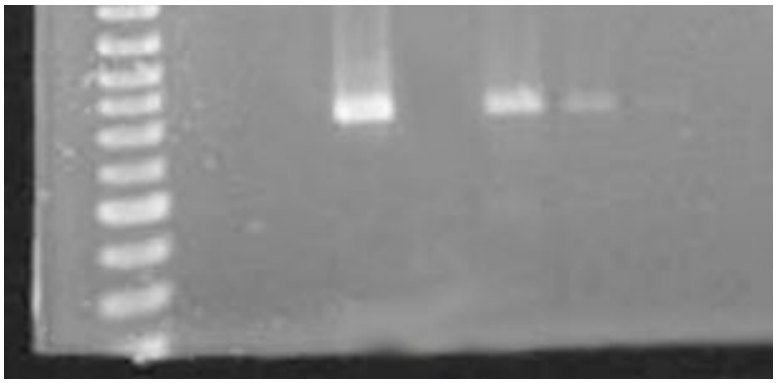


Fig.20. PCR electrophoretogram of *E. durans* strain. Lane 3, positive control; lane 4, negative control; lane 5 and 6, PCD 103.

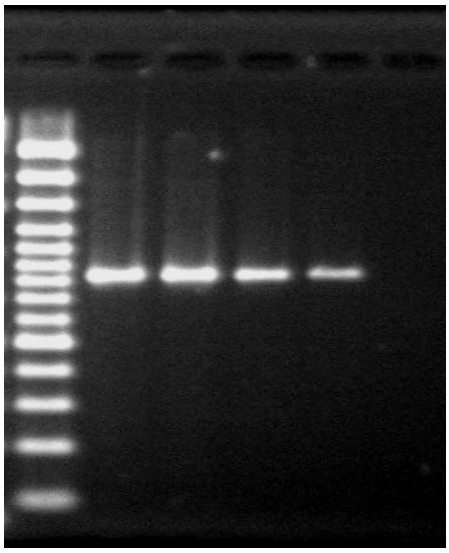


Fig.21. *Bifidobacterium longum*. 831 bp PCR products obtained for *Bifidobacterium* species with *B. longum* specific primers. Lane M, GeneRuler 100 bp DNA Ladder Plus; lane 1, PCB133 [200 ng]; lane 2, PCD733 [200 ng]; lane 3, PCB133 [100ng]; lane 4, PCD733 [100ng]; lane 5, negative control (PCR performed with primers pair BiLON-1/BiLON-2 and *E. coli*).

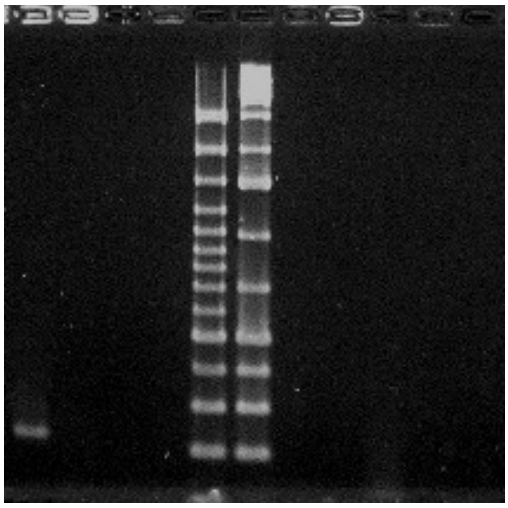


Figure 22. *Lactobacillus pentosus* 250 bp PCR products obtained for 7 *Lactobacillus* species and 1 *Leuconostoc* species with *Lb. pentosus* specific primers. Lane 1, PCA227; lane 2, PCA236; lane 3, PCA263; lane 4, PCA275; lane 5, GeneRuler 1 kb DNA Ladder Plus; lane 6, GeneRuler 100 bp DNA Ladder Plus; lane 7, PCK18; lane 8, PCK73; lane 9, PCK103; lane 10, PCS20; lane 11, negative control (PCR performed with primers pair Lpen-F/16S and *E. coli*).

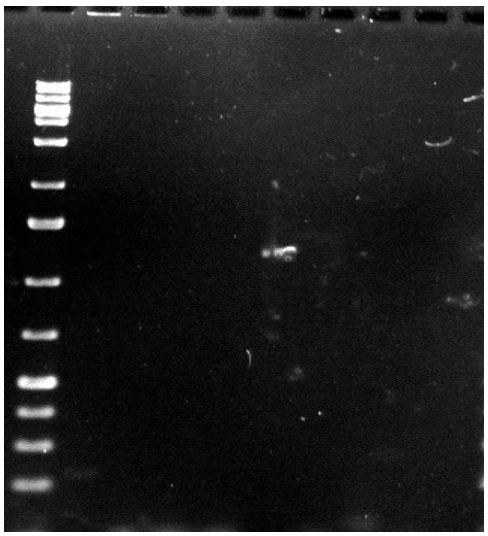


Figure 23. *Leuconostoc mesenteroides*. 1150 bp PCR products obtained for 7 *Lactobacillus* species and 1 *Leuconostoc* species with *Leu. mesenteroides* specific primers. Lane 1, GeneRuler 1 kb DNA Ladder Plus; lane 2, PCA227; lane 3, PCA236; lane 4, PCA263; lane 5, PCA275; **lane 6**, **PCK18**; lane 7, PCK73; lane 8,

PCK103; lane 9, PCS20; lane 10, negative control (PCR performed with primers pair Lmes-F/Lmes-R and *E. coli*).

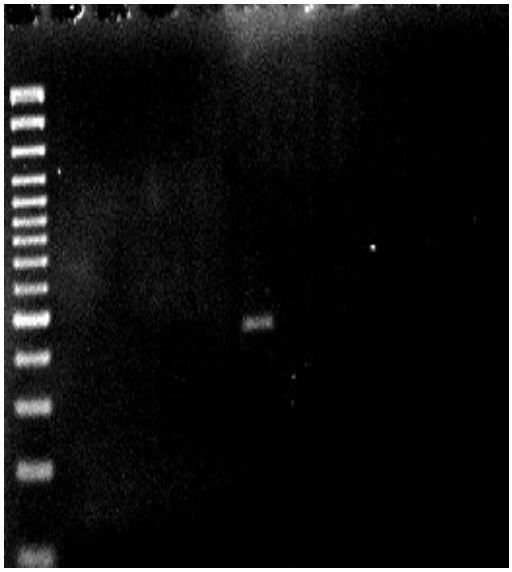


Figure 24. *Lactobacillus delbrueckii* (Tilsala). 500 bp PCR products obtained for 7 *Lactobacillus* species and 1 *Leuconostoc* species with *Lb. dulbrueckii* specific primers. Lane 1, GeneRuler 1 kb DNA Ladder Plus; lane 2, PCA227; lane 3, PCA236; lane 4, PCA263; lane 5, PCA275; **lane 6**, **PCK103**; lane 7, PCK73; lane 8, PCK18; lane 9, PCS20; lane 10, negative control (PCR performed with primers pair Delb-F/Delb-R and *E. coli*).

Chapter 5. Antibiotic resistance

The determination of the antibiotic resistance of bifidobacteria and LAB is an important issue, considering that these probiotics are often co-administered with antibiotics. On the other hand, probiotics can represent a potential source for the spread of antibiotic genes. All strains tested showed resistance to the antibiotic tested: ampycillin, vancomycin, trimethoprim, streptomycin, kanamycin, cefuroxime, chloramphenicol, erythromycin and tetracycline. Further studies are needed to confirm these results.

PART IV

DISCUSSION AND CONCLUSION

Discussion

The beneficial bacterial effects of probiotics on human and animal health have increasingly been highlighted during the past years. According to FAO and WHO guidelines (FAO/WHO, 2002), prospective probiotics must fulfill certain criteria and should be selected through a defined process. The selection criteria for LAB to be used as 'probiotics' include the ability to inhibit the growth of pathogens, to withstand transit through the GI tract, and to survive after food processing conditions. In this context, the aim of the present study was to apply established in vitro tests to evaluate the probiotic potential of selected LAB and bifidobacteria strains.

Among the 60 strains (mainly *Lactobacillus, Pediococcus, Leuconostoc* and *Bifidobacterium* spp.) investigated for their antimicrobial activity by using spot agar test with cell cultures, a relatively high number of them managed to inhibit the growth of *Campylobacter jejuni* strains, an emergent Gram negative pathogen causing human enteritis frequently associated with raw or uncooked poultry products: 60% of the tested strains was able to contrast the growth of *C. jejuni* strain CIP 70.2; 43.3% of *C. jejuni* strain LMG 8842 and 68.3% of *C. jejuni* 221/05 strain. These high percentages might result from the production of organic acids from strain metabolism, which inhibit growth by pH reduction of the medium. In fact, when spot agar test was performer with neutralized supernatants, only 11 strains showed a weak inhibition zone, the majority of which belonging to the genus *Lactobacillus*. The well diffusion assay test performed with neutralized supernatants confirmed that in some strains, in particular PCA 263, PCA 275, PCK 103 (*Lactobacillus* spp.), PCS 20 (unidentified strain isolated from cheese), PCK 73 (unidentified strain isolated from coffee fermentation), PCD 733B and PCK 173 (*Bifidobacterium* spp.), the inhibitory activity may not result only from acidification. The putative presence of non-acidic inhibitory compounds need to be further investigated. A very interesting result is the

higher percentage of inhibition against *C. jejuni* 221/05, the wild strain isolated from chicken GI tract.

Further investigation were aimed at evaluating if the 11 strains showing higher antimicrobial activity against *Campylobacter* spp. were able to survive passage through the gastrointestinal tract. The first barrier is represented by the low pH in the stomach (pH of about 2.5). Since food ingestion can take up to 3 hours, the survival rates of the strain was also examined in artificial gastric juice (pH 2.5) after incubation for up to 3 hours. Two strains, PCS 20 and PCK 103 showed to have strong resistance after 30 minutes, but they lost their viability after this time. The other strains showed lower resistance . The ability of potentially probiotic strains to survive in acidic conditions has been investigated in a number of studies, which have shown great variation between strains and species. Hood and Zottola (1988) showed that no cells of a *Lactobacillus acidophilus* culture were recovered following 45 min exposure to pH 2·0, while at pH 4·0 the number of cells was not significantly reduced after 2 h. Similar trends have been shown for the survival of *Lactobacillus rhamnosus* GG in human gastric juice at pH values ranging from 1·0 to 7·0 (Goldin et al. 1992). In general, *Bifidobacterium* cultures are less acid tolerant than *Lactobacillus* cultures and this can be seen in their reduced tolerance to human gastric juice (Dunne et al. 1999). Furthermore, the choice of the food carrier such as dairy products may enhance microbial survival in gastric juice, most likely due to a buffering or protective effect. This effect may be due to the buffering capacity of the food product. The addition of milk or milk proteins to the gastric juice or media simulating gastric juice significantly increases the pH and enhances survival of some *Lactobacillus* and *Bifidobacterium* species (Charteris et al. 1998). Lactobacilli are mainly acid tolerant or aciduric, particularly when isolated from the harsh environment of the GIT (McLauchlan et al. 1998). This approach has been used in the screening of human faecal *Bifidobacterium* and has yielded strains which are both acid and bile tolerant (Chung et al. 1999): these stressed cultures have a higher ability to survive in the presence of bile and acid compared with other bacteria. So, even if

our strain are less resistants to low pH, they could be used as probiotic cultures because they might show a higher resistance with crossed stresses. Bile-salt resistance is the next major challenge for the microorganisms which are expected to survive in the GIT. Although the intestinal bile acid concentration in human is about 0.3/0.5% (w/v), in this study the tolerance of the bacteria was tested in two different concentrations of 1% and 2% (w/v) of bile salts respectively, since there is limited information about the concentration of bile acid in animal intestine (Kim et al., 2007). With the exception of PCD 733B, PCB 133 and PCK 103, all strains showed tolerance ranging from good to excellent, with a survival percentage reaching 90.71% after 4 hours of 2% bile salts treatment for PCA 227 strain. It is interesting to note that the two strains of Bifidobacteria showed a lower tolerance to stress. After an initial decrease, some strains increased slightly their survival percentage at the maximum time of exposure; this might have been due to the adjustment of the strains to the stress situation. However, remarkable differences between strains are also described in the literature (Berrada et al., 1991; Pochart et al., 1992).

A major challenge associated with the application of probiotic cultures in functional foods and in animal feed is the retention of viability during industrial processing. The control of the resistance of probiotic bacteria to temperature stress may also have potential practical benefits in the industrial fermentation processes in which bacteria with enhanced thermotolerance are required. In this study, we assayed the ability of selected strains to survive after heat treatment with 2 temperatures (50 °C and 55 °C) at different time of exposure. At 50 °C, only PCS 20 and PCB 133 strains showed low/moderate resistance after the longer exposure time (60 min). At 55 °C, these two strains, as well as the PCK 103 strain, showed a reduced heat resistance. The majority of strains retained their viability even at 55 °C and after 60 min, showing a survival percentage ranging from 40% to 89%.

Among the many ways used to preserve food products, the increased osmotic pressure, i.e. the lowering of water activity (a_w), is one of the most widely used. The desiccation or the addition of high amounts of osmotically active compounds such as

salts or sugars lowers the water activity of the food. Therefore, the investigation of the resistance of putative probiotic strains against osmotic stress is of crucial importance in selecting new probiotics for the application in foods and animal feed. In this study, the strains were submitted to 6% NaCl for 30 and 60 minutes, and the results showed the optimal resistance of 10 out of the 11 strains, with a survival percentage higher than 55% after 60 minutes of exposure. Only PCA 263 strains showed low resistance (22.43% after 60 min), probably due to the intrinsic characteristics of the strain.

As to starvation stress, the strains showed variable behaviour. After 6 hours the majority of them showed strong resistance, and the same result was obtained also after 12 hours. After 24 hours, the most resistant strains were PCA 236, PCK 103, PCD 103 and PCS 20 (survival percentage of more than 80%), but also the remaining strains showed resistance even if only moderate. Only PCA 263 strain showed no resistance at all.

The strains tested were also safe, as shown by the absence of the β-hemolytic activity in all the 11 selected strains.

The results from phenotypic identification carried out by using API systems were confirmed by molecular techniques, which showed that the majority of strains belong to the same species *Lactobacillus plantarum* (PCA 236, PCA 263, PCA 275, PCK 73 and PCS 20 strains), while PCB 133 and PCD 733B strains belong to the species *Bifidobacterium longum*. PCA 227 strain was identified as *Lactobacillus pentosus*, and PCK 18 as *Leuconostoc mesenteroides*. PCK 103 as *Lactobacillus delbrueckii* and PCD 103 as *Enterococcus durans*.

As to antibiotic resistance, all strains showed MIC ≥256 μg/ml towards all the antibiotics assayed (Tetracycline, Trimethoprim, Cefuroxime, Kanamycin, Chloramphenicol, Vancomycin, Ampycillin, Streptomycin and Erythromycin), thus evidencing a high resistance of all the strains. Several studies are present in the literature regarding the antibiotic resistance of *L. plantarum* (West and Warner, 1985; Florez et al. 2006; Engervam et al. 2009); these works evidence that MIC values for

several antibiotics, among which those tested in this research, vary widely between strains. A high percentage of *L. plantarum* strains described in the literature are resistant to tetracycline, erythromycin, and cloramphenichol and most of the antibiotic resistance strains of *L. plantarum* harboured plasmid-encoded resistance genes (Egervan et al. 2009). Therefore, when present in the food chain and in the intestinal tract of animals and humans, these bacteria may function as reservoir of antibiotic resistance genes than can be transferred to pathogenic bacteria. The determination of the transferability of the antibiotic resistance carried by the strains characterized in this work is therefore essential to assay the safety of the strains.

Conclusion

The *in vitro* criteria used in this study for the selection of candidate probiotics have been chosen from the selection guidelines provided by the FAO/WHO committee. The eleven putative probiotic bacteria strains (identified by both phenotypical and molecular methods as 5 *L. plantarum* strains, a *L. pentosus* strains, a *L. delbrueckii* strain, an *Enterococcus durans* strain, a *Leuconostoc mesenteroides* strain and 2 *Bifidobacterium longum* strains) are able to inhibit the growth of *C. jejuni* strain and are resistant to the stresses in the GI tract, with the exception of low pH. Low resistance to acidic pH can be overcome by the choice of a food carrier or by protection via microencapsulation that may enhance microbial survival in gastric juice. The strains survive well in food processing conditions (heat, osmotic and starvation stress) and have shown no β-hemolytic activity. The antibiotic resistance of these strains needs further characterization. However, we can conclude that the selected strains, in particular PCA 236, PCA 275, PCD 103, PCB 133 strains could be selected as probiotics to be used in industry, but also in nutraceutical form, provided they are protected from the action of low pH.

The Pathogen Combat Project

The research described in this thesis is framed within EU 6th Framework Programme (www.pathogencombat.com) PathogenCombat. It is an integrated project that began on 1st of April 2005 and will run until April 2010. PathogenCombat consists of 44 partners in Europe and Australia: 24 are research institutions and organisations, 17 are SMEs and 3 are industrial partners. Food safety is of fundamental importance to the European consumer, the food industry and the economy. The impact on trade and competitiveness is substantial. Despite the significant investment in the field , the incidence of food derived diseases is increasing in the EU. PathogenCombat aims at dealing with this pan-European problem through a holistic, multidisciplinary approach towards threats from new/emerging pathogens in the entire food chain. A number of advanced platforms will be developed to investigate the survival and virulence expression of pathogens in feed and food and on contact surfaces in the food chain including the intestinal tract of farm animals. The platforms, several of which are used for the first time in food safety studies, include bioimaging, laser tweezers, phage display/convergent evolution, functional mammalian cell models, functional genomics and microarrays. New/emerging foodborne bacteria, yeast, filamentous fungi and viruses are targeted for milk and dairy products, ruminants, poultry and pigs and their meat products. The overall and specific objectives of PathogenCombat can briefly be described as follows:

- The production of safe food with no or acceptably low levels of pathogens.
- The determination of factors in the food chain, which enable the viability, persistence and virulence of pathogens.
- The detection and prediction of the occurrence and virulence of pathogens in the food chain with molecular biology based culture independent techniques and microarrays.

- The determination of host-pathogen interaction with functional cell model replacing the use of experimental animals.
- The prevention of pathogen transmission along the food chain through new processing technologies and systems, protective cultures and new information on host-pathogen interaction.
- The application of PathogenCombat deliverables. in the food chain/SMEs.
- The control of pathogens throughout the food chain with new mathematical models.
- The development of a Food Safety Management System, which incorporates the deliverables of PathogenCombat.
- The creation of a Small and medium Enterprises (SME) Network including dissemination of knowledge, dissemination of results and training of SMEs and consumer awareness of food safety.

In particular, inside the PathogenCombat, the aim of WorkPackage 10 is to find lactic acid bacteria and bifidobacteria strains able to inhibit, either at the level of farm animals or at the level of food products, the pathogens studied in PathogenCombat. Specifically, to obtain a selection of well characterized protective and probiotic cultures of lactic acid bacteria and bifidobacteria strains, which display a clear inhibition of the pathogenic bacteria and mycotoxin producing moulds considered in the project and which can survive the passage of the gastrointestinal tract and food processing conditions and environments. Strains with this potential can then be considered as protective and probiotic cultures to be incorporated in the development of prevention strategies for foodborne pathogenic microorganisms throughout the food chain.

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