

# Antibiotic Susceptibility of Probiotic Bacteria

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## 1. Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria widely distributed in nature. These bacteria are found in gastrointestinal (GI) and urogenital tract of humans and animals; they are present on plant material, in milk and meat, and numerous fermented foods. Lactic acid bacteria have been associated with traditional dairy products, cereals, vegetable and meat fermented foods, due to their natural presence leading to spontaneous fermentation. They are also used as starter cultures in industrial food production, as well as in the production of probiotic products due to their potential health benefits to consumer. Milk and dairy products are the most examined food system for the delivery of probiotic bacteria to the human gut. The probiotic concept has progressed and is now in the focus of different research. Significant improvements have been made in selection and characterization of new cultures and their application in food production.

The food products, which are produced by traditional methods, exhibit a rich biodiversity with the respect to bacterial contents. From these products, new probiotic strains with the potential functional properties can be isolated and selected. The selected strains have to be further characterized in order to be used in the food industry. Before the probiotics can benefit human health, they must fulfill several criteria including: a) scientifically validated health properties; b) good technological properties meaning that they can be manufactured and incorporated into food products without losing viability, functionality and technological performance; c) high survival through the upper gastrointestinal tract and high viability at its site of action; d) antagonistic activity to pathogens; e) antibiotic susceptibility; and f) to be able to function in the gut environment. Bearing in mind importance of antibiotic resistance of LAB in food chain, antibiotic susceptibility of potential probiotic strains is a very important criteria for their selection.

In the recent decade, releasing of antibiotics in biosphere seriously increased, leading to a strong selective pressure for the emergence and persistence of resistant LAB strains. Since LAB are naturally present in traditionally made fermented food and GI tract and are also added as starter culture or probiotic bacteria in industrial food production, concerns have been raised about the antibiotic resistance of these beneficial bacteria strains. Probiotic bacteria can help maintaining balance in gastrointestinal tract in cases of diarrhea caused by antibiotic treatment. However, there is high risk associated with the ability of these resistant

strains to transmit the resistance gene to pathogenic bacteria in gut microbiota. This can complicate the treatment of a patient with an antibiotic resistant bacterial infection or disease. The circulation of genes coding for antibiotic resistance from beneficial LAB in the food chain via animals to humans is a complex problem. Therefore, there is a need to evaluate the safety of potential probiotic strains regarding their ability to acquire and disseminate antibiotic resistance determinants in selection of LAB.

In this study, importance of LAB in the food chain will be reviewed. Morphological and biochemical characteristics of lactobacilli, bifidobacteria and enterococci, as well as criteria for probiotic selection and role of probiotics in health benefit will be discussed. Antibiotic susceptibility as criteria for potential probiotic bacteria selection and mechanisms of gene transfers will be considered.

## **2. Lactic acid bacteria in the GI tract**

The human GI tract represents a complex ecosystem in which interactions between food, microbes and the host cells occur. The bacterial population of normal gut of an adult comprise of more than 500 different species. The quantity of microbes present in the intestine (about  $10^{14}$ ) exceeds 10-fold the total number of all human cells (Backhed et al., 2005). The most important function of this intestinal microbiota is to act as a microbial barrier against pathogens, by so-called competitive exclusion mechanisms, but also influence the humoral and cellular mucosal immune responses during the neonatal phase of life, and thereafter to maintain a physiologically-normal steady-state condition throughout life (Tancrede, 1992). The gut microflora profoundly influences nutritional, physiologic and protective processes. Both direct and indirect defensive functions are provided by the normal microbiota. Specifically, gut bacteria directly prevent colonization by pathogenic organisms by competing for essential nutrients or for epithelial attachment sites. By producing antimicrobial compounds, volatile fatty acids, and chemically modified bile acids, indigenous gut bacteria also create a local environment that is generally unfavourable for the growth of enteric pathogens. This phenomenon is called Colonization Resistance, which can be defined as the ability of microorganisms belonging to the normal gut microflora to impede the implantation of pathogens (van der Waaij, 1988). This function of the microflora is also known as the barrier effect. While probiotic bacteria improve colonization resistance, consensus thinking is that the importance of LAB as probiotic agents lies more in the indirect mechanisms such as immunomodulation. When the genetic repertoire of these bacteria is considered, the GI tract translates into a reservoir of genes encoding numerous physiological functions from which the human GI tract can benefit. Bacteria represent the most extensively investigated group of microorganisms. Which species of bacteria will be long-term colonized in GI tract depends on the biochemical capability of the microorganisms, the microenvironment determined by the host cells and the available foodstuffs. Lactobacilli are probably the most well known representative of favourable microorganisms in GI tract. There is a number of species of lactobacilli reside in the human intestine in a symbiotic relationship with each other and with other microorganisms (Claesson et al., 2007). They are generally considered essential for maintaining gut microfloral health; however, it is the overall balance of the various microorganisms which is ultimately of most importance.

## 2.1 The composition of the GI tract

The composition of the GI microflora undergoes considerable changes from the day of birth until adulthood. The GI tract of a normal fetus is sterile, but colonization begins immediately after birth and is influenced by the mode of delivery, the infant diet, hygiene levels and medication (Benett et al., 1986; Gronlund et al., 1999). During the first months of life diet has a significant influence on the development of the intestinal flora (Heavey & Rowland, 1999; Stark & Lee, 1982). Within one to two days facultative anaerobes predominate and create a reduced environment that allows the growth of strict anaerobes. Within three to four days, bifidobacteria appear and become predominant. The average number of bifidobacteria in breast-fed infants is  $10^{10}$ – $10^{11}$  cfu/g. In formula fed infants, bifidobacteria have also been demonstrated to be a numerically important species, but they generally occur in lower numbers than in breastfed infants of the same age (Mountzouris et al., 2002). The predominance of beneficial bacteria in the gut microbiota of breast-fed infants is thought to result from the fermentation of oligosaccharides-non-digestible carbohydrates consisting of several linked monosaccharides (typically 3-10 simple sugars) in breastmilk (Agostoni et al., 2004). Oligosaccharides pass unabsorbed through the small intestine into the colon, where they are fermented by resident bifidobacteria to short-chain fatty acids and lactic acid, reducing the gut pH to approximately 5.7, thus providing the protection against enteric infections (Newburg, 2000, Coppa et al., 2004). In contrast, the gut microflora of formula-fed infants produces a different profile of short-chain fatty acids and a pH in the local microenvironment of approximately 7.0 (Ogawa et al., 1992). Infant faecal flora appears to be stabilized at 4 weeks of age and until weaning when introduction of solid foods takes place (Mountzouris et al., 2002). During weaning, bifidobacteria decrease by 1 log, the microbiota alters from infant-type to adult-type, and a remarkable proliferation of bacteroides, eubacteria, peptostreptococcaceae, and clostridia occur. The faecal flora of children closely resembles that of adults, where the numbers of bacteroidaceae, eubacteria, peptococcaceae, and usually clostridia outnumbered bifidobacteria, which constitute 5-10% of the total flora.

An adult individual's GI tract is extremely stable and it is very difficult to introduce new species. There are several factors that can alter the composition of the GI flora such as medications (especially antibiotics), diet, climate, aging, illness, stress, pH, infections, geographic location, and even race (Murphy et al., 2009). Thus it is not surprising to find out that the composition of the GI flora not only differs among individuals, but also differs during the life within the same individual. Furthermore, indigenous bacteria are not distributed randomly throughout the gastrointestinal tract but instead are found at population levels and in species distributions that are characteristic of specific regions of the tract.

As shown in Figure 1, there are three main regions that offer very different conditions for the survival of various microorganisms in the GI environment. In the stomach, microbial growth is greatly reduced by the high acidity and presence of oxygen provided by the swallowing. As a result, in the stomach acidotolerant microorganisms and facultative anaerobes such as lactobacilli, streptococci, yeasts, etc. are present. In the second region (small intestine), the microflora consists mainly of facultative anaerobic bacteria such as lactobacilli, streptococci and enterobacteria, and anaerobes such as bifidobacteria, bacteroides and clostridia. In the last region (colon), the number of bacteria is considerably high, due to the low redox potential and relatively high concentration of short-chain fatty acid, and counts  $10^9$ – $10^{12}$  cfu/ml (Cummings et al. 1989). The colon has an important role in

food digestion, and microflora in this region participates in the transformation of many carbohydrates, proteins and amino acids. The microflora of the colon is very complex and dominated by anaerobic bacteria (*Bacteroides* spp., *Clostridium* spp., *Bifidobacterium* spp., etc), while the facultative anaerobic bacteria are less numerous and represented by lactobacilli, enterococci, streptococci and *Enterobacteriaceae*. Yeasts (eg., *Candida albicans*) are relatively poorly represented.

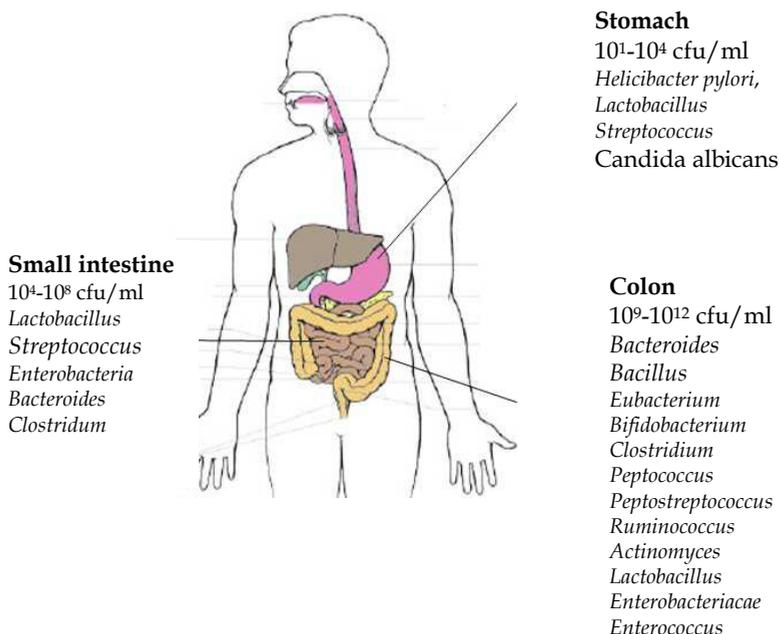


Fig. 1. The average concentration of microorganisms in the GI tract (adapted from Ouwehand & Vesterlund, 2003)

As part of gut microbiota, it is estimated that lactobacilli are present in following concentrations:  $10^3$ - $10^6$  cfu/ml in the oral cavity;  $10^3$  cfu/ml in the stomach;  $10^4$  cfu /ml in the duodenum and jejunum;  $10^8$  cfu /ml in the ileum and  $10^9$  cfu /ml in the colon (Reuter, 2001; Koll et al., 2008; Ryan et al., 2008). An overview of lactobacilli commonly found in the GI tract microbiota is shown in Table 1.

Considering that lactobacilli and bifidobacteria constitute a significant population in the GI tract, this environment represents a good source for the isolation of new strains of LAB.

### 3. Lactic acid bacteria as probiotic bacteria

By definition probiotics are described as „living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition“ (Guarner & Schaafsma, 1998). Regarding the probiotics, the majority of research is focused on bacterial genera *Lactobacillus* and *Bifidobacterium* (Table 2).

Oral cavity	Stomach	Small intestine	Faeces	Colon epithelial biopsies
<i>L. paracasei</i>	<i>L. gasseri</i>	<i>L. gasseri</i>	<i>L. gasseri</i>	<i>L. plantarum</i>
<i>L. rhamnosus</i>	<i>L. reuteri</i>	<i>L. reuteri</i>	<i>L. paracasei</i>	<i>L. rhamnosus</i>
<i>L. fermentum</i>	<i>L. ruminis</i>	<i>L. rhamnosus</i>	<i>L. ruminis</i>	<i>L. paracasei</i>
<i>L. plantarum</i>			<i>L. reuteri</i>	
<i>L. gasseri</i>			<i>L. plantarum</i>	
			<i>L. salivarius</i>	
			<i>L. sakei</i>	

Table 1. *Lactobacillus* species distribution in different parts of the GI tract (Lönnermark, 2010)

<i>Lactobacillus</i> sp.	<i>Bifidobacterium</i> sp.	<i>Enterococcus</i> sp.	Others
<i>L. acidophilus</i>	<i>B. bifidum</i>	<i>E. faecium</i>	<i>Lactococcus lactis</i> ssp. <i>lactis</i>
<i>L. plantarum</i>	<i>B. infantis</i>	<i>E. faecalis</i>	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>
<i>L. casei</i>	<i>B. adolescentis</i>		<i>Leuconostoc mesenteroides</i>
<i>L. rhamnosus</i>	<i>B. longum</i>		<i>Pediococcus acidilactici</i>
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	<i>B. breve</i>		<i>Propionibacterium freudenreichii</i>
<i>L. fermentum</i>	<i>B. lactis</i>		<i>Streptococcus thermophilus</i>
<i>L. johnsonii</i>			
<i>L. gasseri</i>			
<i>L. salivarius</i>			
<i>L. reuteri</i>			

Table 2. Lactic acid bacteria used as probiotics (adapted from Gardnier et al., 2002)

They have a reputation of health promoters and they have a significant role as probiotic bacteria in the production of different foods, particularly in the production of fermented dairy products. Central position of lactobacilli and bifidobacteria in probiotic formulation is argued due to (a) the association of these bacteria with human health, (b) the fact that they possess the Generally Regarded As Safe (GRAS) status. Enterococci, although not GRAS organisms, have been used as probiotics too. Some other LAB such as lactococci, pediococci, *Leuconostoc*, propionibacteria have also received attention as potential probiotic cultures (Table 2).

### 3.1 *Lactobacillus*

The genus *Lactobacillus* is large heterogeneous group of microorganisms, which lacks catalase and cytochromes and is usually microaerophilic, with growth improved under anaerobic conditions (Kandler & Weiss, 1989). They are Gram-positive nonmotile rods, often in pairs or chains, coccobacilli to long rods. They have a strictly fermentative metabolism and convert glucose solely or partly to lactic acid. They are classified as homofermentative (producing mainly lactic acid) or heterofermentative (producing carbon dioxide, ethanol, acetic acid and lactic acid). The optimum growth temperature is in the mesophilic range (30-40°C), but some strains can grow below 15°C and some at temperatures up to 55°C. Differentiation of *Lactobacillus* species depends on physiological criteria, carbohydrate fermentation, biochemical and molecular characterization (Petrovic et al., 2006). Lactobacilli

play crucial role in the production of fermented foods: vegetables, meats and dairy products. Non starter lactic acid bacteria (NSLAB) are lactobacilli which form significant proportion of the microflora of most cheese varieties during ripening. Many species of mesophilic *Lactobacillus* have been isolated from cheese; the ones most frequently encountered are *L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. curvatus* (Beresford et al., 2001). For example, *L. plantarum*, *L. casei* ssp. *casei* and *L. brevis* have been isolated from Armada cheese, a Spanish goat milk cheese (Herreros et al., 2003), *L. plantarum*, *L. paraplantarum*, *L. paracasei* ssp. *tolerans*, *L. sake*, *L. curvatus* and *L. pentosus* from Batzos, a traditional Greek cheese made from raw goat's milk (Psoni et al., 2003) and *L. para.paracasei*, *L. plantarum*, *L. curvatus* and *L. brevis* from Sjenica cheese, traditional Serbian white brined cheese (Radulović, 2010). Traditional homemade dairy products have great potential for isolation of new strains, which could be used as starter cultures in food industry, as adjunct cultures for improving flavour of cheeses, or as probiotic in production of functional foods.

### 3.2 *Bifidobacterium*

Species from the genus *Bifidobacterium* are generally characterized as Gram-positive, non-spore forming, nonmotile and catalase negative. They are strict anaerobes, although some species and strains may tolerate oxygen in the presence of carbon dioxide. Within the genus *Bifidobacterium* pleomorphism exists and it is described as short regular, thin cells with pointed ends, long cells with slight bends with a large variety of branching; single or in chains of many elements; in star-like clusters or disposed in "V" or palisade arrangements (Scardovi, 1986). In bifidobacteria, glucose catabolism occurs through the fructose 6-phosphate phosphoketolase pathway, which can be used as distinguishing feature of bifidobacteria. During fermentation, acetic and lactic acids are produced in molar ratios 3:2. The optimum temperature for growth is 31-41°C within the range 25-45°C. Bifidobacteria are less acid tolerant than lactobacilli and no growth occurs at pH values less than 4.5 (Scardovi, 1986). Nutritional requirements for growth of bifidobacteria are less complex than those of lactobacilli, but in some cases bifidobacteria do require specific factors for optimal growth (Modler et al., 1990). Bifidobacteria are natural habitants of GI tract and strains with probiotic properties are mainly of human origin. *Bifidobacterium* species constitute a significant portion of probiotic cultures used in functional food production.

### 3.3 *Enterococcus*

Although lactobacilli and bifidobacteria are most commonly used as probiotics, some enterococci can also be used as health-promoting bacteria. Species *E. faecium* and *E. faecalis* have been used as probiotic bacteria, although they are not recognized as GRAS organisms. All species of genus *Enterococcus* are Gram-positive, non-spore forming, catalase negative, facultative anaerobes. They are spherical or ovoid cocci in pairs or short chains. Their properties, such as the ability to grow at 10°C and 45°C in 6.5% NaCl and at pH 9.6 and their survival heating to 60°C for 30 min, are used to differentiate enterococci from other Gram-positive catalase-negative cocci (Franz et al., 1999). Enterococci are homofermentative with respect to glucose metabolism, although some amounts of formic and lactic acid may be produced in some media. As the other LAB, enterococci do require B vitamins, amino acids, purine and pyrimidine bases for optimal growth (Garg & Mital, 1991).

### 3.4 LAB associated with therapeutic properties

In recent years an increasing number of probiotic pharmaceutical preparations as well as food supplements are being promoted with health claims. The application of LAB has been more developed for the production of functional foods, where probiotic bacteria have an important role. The commercial probiotic strains, used in functional food production, must be well-substantiated with scientific evidence.

The health benefits of probiotic bacteria can be considered as nutritional or therapeutic/prophylactic. Nutritional benefits are mainly connected with their role in enhancing the bioavailability of vitamins and minerals, and an increase of the digestibility of protein (Tamime et al., 2003). Many researchers (Begley et al., 2006; Ouwehand et al., 2003; Rodriguez et al., 2010) investigated the therapeutic/prophylactic benefits of probiotics and confirmed their effects:

- Prevention of diarrhoea caused by certain pathogenic bacteria and viruses;
- Regulation of intestinal microflora after an antibiotic therapy;
- Treating the infection with *Helicobacter pylori*, responsible for the development of gastritis, ulcers and gastric cancer;
- Improvement of digestion in "lactose-intolerant" individuals, who have reduced capability for lactose digestion;
- Anticancer effect, as a result of the production of certain compounds during its growth;
- Reducing cholesterol levels;
- Stimulation of  $\gamma$ -interferon production, which contributes to increased resistance to some infections;
- Increasing antibody titer (IgG immunoglobulins), which enhances the immune response of an organism;

Health effects are related to microflora modification and strengthening of the gut mucosal barrier. Some scientific data indicated that probiotic strains have potential in the prevention and treatment of intestinal and urogenital infections and these cultures may be useful as an alternatives to antibiotic therapy. The World Health Organization (WHO) has recommended the reconsideration of microbial interference therapy for infection control (Bengmark, 1998). Certain probiotic strains have been shown multiple effects including prevention of pathogen attachment and invasion in cell culture, inhibiting of the growth of enteropathogens *in vitro* and enhancement the immune response. Considering these effects, the usage of probiotics may decrease antibiotics dependence. Fooks et al., (1999) have suggested that probiotic bacteria can control the infection in several ways such as the competition for nutrients, secretion of antimicrobial substances, reduction of pH, blocking of adhesion sites, reduction of virulence, blocking of toxin receptor sites, immune stimulation and suppression of toxin production.

Infections by bacterial or viral agents most frequently result in diarrhea. One of the most investigated probiotic, *L.rhamnosus* GG, has been very effective in the treatment of viral diarrhea in children, most cases of which were caused by rotavirus. Other probiotic strains such as *Lactococcus casei* Shirota, *B. bifidum* and *S. thermophilus* have also been shown to be effective in the treatment and prevention of rotavirus diarrhea in children (Korhonen, et al., 2007; Saavedra et al., 1994).

Antibiotic-associated diarrhea (AAD) is a major clinical problem that occurs following antibiotic use. The most serious form of this kind is pseudomembranous colitis. Diarrhea is caused by pathogen overgrowth and in 20% cases the etiological agent is *Clostridium difficile*, a pathogen that is especially persistent and difficult to treat (Lewis & Freedman, 1998). Antibiotics are often used to treat for pseudomembranous colitis or other AAD, although the relapse may occur when the probiotic therapy may be especially useful. Oral therapy with *L. rhamnosus* GG was effective in the prevention of AAD, in treatment of colitis, as well as in traveller's diarrhea (Shah, 2007). A combination of *B. bifidum*, *S. thermophilus*, *L. delbrueckii* spp. *bulgaricus* and *L. acidophilus* have been also effective in the prevention of traveller's diarrhea (Black et al., 1989).

On the other hand, many studies have shown no effect of probiotic treatment (Lewis & Freedman, 1998). There is still some doubts regarding the quality and efficacy of probiotic products. Nevertheless, the probiotic food industry is flourishing. In many European countries the market is expanding resulting in sell of probiotic yogurts that account for over 10% of all yogurts sold in Europe (Stanton et al., 2001).

#### 4. Criteria associated with probiotic bacteria

Consortium of LAB constitute a major part of the natural microflora of human intestine and when present in sufficient numbers create a healthy equilibrium between beneficial and potentially harmful microflora in the gut (Dune et al., 2001). For some positive effects on human health, a probiotic strain has to reach the large intestine at a concentration of about  $10^7$  viable cells/g (Stanton et al., 2001). Microorganisms ingested with food begin their journey to the lower intestinal tract via the mouth and are exposed during their transit through the GI tract to successive stress factors that influence their survival. The time from entrance to release from the stomach is about 90 min, but further digestive processes have longer residence times (Berrada et al., 1991). Probiotic bacteria must overcome physical and chemical barriers in the GI tract, especially acidic environment of the stomach, and then the activity of hydrolytic enzymes and bile salts in the small intestine. In a typical acid tolerance tests, the viability of potential probiotic organisms is determined by exposing them to low pH in a buffer solution or medium for a certain period of time, during which the number of surviving probiotic bacteria is determined. The generally requirements for probiotics are shown in Table 3.

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Acid and bile stability
Adherence to human intestinal cells
Ability to reduce the adhesion of pathogens to surfaces
Colonization of human GI tract
Antagonism against carcinogenic and pathogenic bacteria
Production of anti-microbial substances
Survive the various technological processes of production
Safety evaluation: nonpathogenic, nontoxic, nonallergic, nonmutagenic
Desirable metabolic activity and antibiotic resistance/sensitivity
Clinically validated and documented health effects

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Table 3. The desired properties of probiotic strains (adapted from Mattila & Sarraela, 2000)

Bile plays an essential role in lipid digestion; it emulsifies and solubilizes lipids and functions as biological detergent. Prior to secretion into the duodenum, bile acids, which are synthesized from cholesterol, are conjugated to either glycine or taurine in liver (Begley et al., 2006). In the colon conjugated bile undergoes to the various chemical changes including deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation, almost solely by microbial activity (Begley et al., 2006). Bile reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids and these modifications may affect not only the cell permeability and viability, but also the interactions between the membranes and the environment. Bile salt hydrolases (BSHs) are generally intracellular, oxygeninsensitive enzymes that catalyze the hydrolysis of bile salts (Liong & Shah, 2005). A number of BSHs have been identified and characterized in probiotic bacteria (Franz et al., 2001a).

The adhesion ability as well as interaction with pathogens is regarded as important selection criteria for potential probiotic strains (Salminen et al. 2010). Adhesive properties of LAB depend on a variety of factors, including non-specific adhesion determined by electrostatic or hydrophobic forces and specific binding dependent on particular molecules. To examine the adhesive property of LAB, several models have been developed. These include binding to tissue culture cells (Tuomola & Salminen, 1998), radiolabelling (Bernet et al., 1993), intestinal mucus (Ouweland et al., 2001), extracellular matrix proteins (de Leeuw et al., 2006) and resected colonic tissue (Vesterlund et al., 2005). Although none of these models reflect the complex interactions occurring in the mucosal layer of the digestive tract, they represent a rapid method for the screening of potential probiotic strains.

Other functional property used to characterize probiotics is the production of antimicrobial compounds. Several antimicrobial substances that have considerable advantages in competition with pathogens and other harmful bacteria are produced by LAB (Klare et al., 2007; Radulović et al., 2010a). These substances include fatty acids, organic acids, hydrogen peroxide, and diacetyl, acetoin and the most studied inhibitory peptides called 'bacteriocins' (Todorov et al., 2011). The ability of probiotics to establish in the GI tract is enhanced by their ability to eliminate competitors. Some examples of antimicrobial substances produced by probiotic bacteria are presented in Table 4.

Probiotic	Compound
<i>Lactobacillus</i> GG	Wide spread antibiotic
<i>L. acidophilus</i>	Acidolin, Acidophilin, Lactocidin
<i>L. delbrueckii ssp. bulgaricus</i>	Bulgarican
<i>L. plantarum</i>	Lactolin
<i>L. brevis</i>	Lactobacillin, Lactobrevin
<i>L. reuteri</i>	Reuterin

Table 4. Antimicrobial substances (Fuller, 1992)

Antibiotic susceptibility of potential probiotic strains is also considered as an important selection criterion for potential probiotic status (Hummel et al., 2007). Some LAB may carry potentially transmissible plasmid-encoded antibiotic resistance genes. The transmission of antibiotic resistance genes to unrelated pathogenic or potentially pathogenic bacteria in the gut is a major health concern related with the probiotic application (see detailed information in paragraph 6).

Along with above mentioned criteria, selected probiotic strains have to be able to survive well in the food and to have the appropriate technological properties (e.g. acidification during fermentation if required). In addition, it is important that the added probiotic does not adversely affect the taste, smell, and texture of the food or beverage.

*In vitro* tests based on these selection criteria, although not a definite means of strain selection, may provide an useful initial information. A validation model system, such as a Simulator of the Human Intestinal Microbial Ecosystem (SHIME), which aims to mimic complex physiological and physiochemical *in vivo* reactions, may also be of value in strain selection. However, the ultimate proof of probiotic effects requires validation in well designed statistically sound clinical trails. Generally, tools that may be employed in such an assessment include *in vitro* studies, studies of strain properties, pharmacokinetic studies, animal studies, use of intestinal models, human studies and epidemiological surveillance. Each strain needs to be tested separately.

It is evident that the selection of new potential probiotic bacteria is an enormous and time-consuming task with uncertain results.

## 5. Antibiotic resistance of LAB in the food chain

During the recent years, there has been great concern about the possibility of spreading the antibiotic resistance in the environment. According to the European Commission (2005), it has been estimated that one to ten million tons of antibiotics has been released into the biosphere over the last 60 years. This has lead to very strong selective pressure for the emergence of resistant bacterial strains. Since LAB are present in the GI tract in large amounts, LAB resistant to certain antibiotics could benefit the host organism. Nevertheless, there is a risk associated with the ability of these resistant strains to transmit the resistance gene to pathogenic bacteria. Literature data pointed out that some LAB, the predominant microbiota in fermented dairy and meat products, may serve as reservoirs of antibiotic resistance genes potentially transferable to human pathogens (Mathur & Singh, 2005).

The food chain could be regarded as one of the main pathways for the transmission of antibiotic resistant bacteria from animals to humans (Singer et al., 2003). Molecular analysis of resistance genes localized on transferable genetic elements showed they are identical in humans and animals, which confirm that food of animal origin, particularly sausages and cheeses made from raw milk, serve as a vehicle for the transmission of resistant bacteria and antibiotic resistance determinants. The antibiotics application in sub-therapeutic levels in animal's drinking water and feed increases the selective pressure and amplify the transfer of antibiotic resistance between bacterial species. Thus there is a direct correlation between the indigenous microflora of the GI tract of animals with the GI tract of humans. Although many species of LAB used as starter and probiotic cultures possess GRAS status, potential risk to human health caused by the genes transfer of antibiotic resistance has not yet been fully defined. To address this aspect, the safety of LAB should be verified with the respect of their ability to acquire and disseminate resistance determinants (Kastner et al., 2006). Particular concern is due to the evidence of a widespread occurrence in this bacterial group of conjugative plasmids and transposons. The presence of transmissible antibiotic resistance markers in the safety evaluation of LAB strains is a very important task since genes conferring resistance to several antimicrobials (e.g. chloramphenicol, erythromycin,

streptomycin, tetracycline, and vancomycin) located on transferable genetic elements (plasmids or transposons) have already been characterized in lactococci (Perreten et al., 1997), lactobacilli (Axelsson et al., 1988; Danielsen, 2002) and enterococci (Eaton & Gasson, 2001; Huys et al., 2002) isolated from food. However, the transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of human GI tract and hence to pathogenic bacteria, has not been fully addressed.

Irrespective of the antibiotic resistance mechanisms and the bacterial taxon involved, the possibility of spreading an antibiotic resistance determinant through horizontal transfer relies on its genetic basis. Therefore, a distinction between intrinsic and acquired resistance has to be made. Antibiotic resistance may be intrinsic for bacterial species or a genus, and it is characterized by the ability of an organism to survive in the presence of certain antimicrobial agents, due to its inherent characteristics of resistance. Intrinsic or “natural” resistance mechanisms involve the absence of the target, low cell permeability, antibiotic inactivation and the presence of efflux mechanisms. Enterococci are intrinsically resistant to cephalosporins and low levels of aminoglycoside and clindamycin (Teuber et al., 1999). Lactobacilli, pediococci and *Leuconostoc* spp. have been reported to have a high natural resistance to vancomycin, a property that is useful to separate them from other Gram-positive bacteria (Hamilton-Miller & Shah, 1998; Simpson et al., 1988). Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen & Wind, 2003). For a number of lactobacilli a very high frequency of spontaneous mutation to nitrofurazone ( $10^{-5}$ ), kanamycin and streptomycin was found (Curragh & Collins, 1992). From these data it is clear that inter-genus and inter-species differences exist, and consequently identification at species level is required in order to interpret phenotypic susceptibility data. Acquired resistance is a characteristic of some strains within a species usually susceptible to certain antibiotics and can be horizontally spread among the bacteria. The acquisition of antibiotic resistance occurs via the mutation of pre-existing genes or by horizontal transmission of resistance determinants. With some exception, intrinsic resistance and resistance by mutation are unlikely to be disseminated, although any gene responsible for intrinsic resistance may spread provided that it is flanked by insertion sequences (European Commissions); horizontally transferred genes, particularly those carried on mobile genetic elements, are those most likely to be transmitted (Normark & Normark, 2002). Among the three well-known mechanisms for horizontal gene exchange between bacteria, namely free DNA mediated transformation, bacteriophage induced transduction, and conjugation, the last is acknowledged to be the most relevant for antibiotic resistance gene transfer (Salysers, 1995). Resistance genes are frequently carried by the mobile genetic elements involved in these mechanisms, such as plasmids and conjugative transposons, which can be freely exchanged irrespective of genus or species barriers, resulting in resistance transfer or co-transfer (Levy, 1986). Recently it has been discovered that the so-called “mobilome” also involves other genetic elements: the transposon can carry integrons, which are not self-transmissible but carry a gene encoding an integrase, which in turn mobilises resistance genes borne on the integron as cassettes (Clementi & Aquilanti, 2011). This mechanism seems to be active only in the context of resistance gene exchange and it surely determines a substantial increase in the horizontal mobility of these genes.

### 5.1 Procedure for antimicrobial susceptibility/resistance patterns of LAB

As mentioned above, an intrinsic resistance and resistance by mutation are unlikely to be disseminated, so the risk is mainly characterized by horizontally transferred genes. Therefore, distinction between natural and acquired antibiotic resistance among the population of LAB is of a great importance. Analysis of Minimal Inhibitory Concentration (MIC) and their distributions in defined species/antibiotic combinations helps to differentiate between these two resistance mechanisms. When a bacterial strain demonstrates higher resistance to a specific antibiotic than the other strains of the same taxonomical unit, the presence of acquired resistance is indicated and there is a need for further analysis to confirm the genetic basis of resistance.

According to Murray et al., (2003) the MIC distribution of a given antibiotic for a single bacterial species in the absence of resistance mechanisms should approach statistical normality while bimodal distribution of MIC values suggest acquired resistance. For the purpose of identifying bacterial strains with acquired and potentially transferable antibiotic resistance, microbiological breakpoints have been defined. Microbiological breakpoints are set by studying the MIC distribution in the bacterial population and the part of population that clearly deviates from a susceptible majority is considered resistant (Olsson-Liljequist et al., 1997).

The data used for the definition of microbiological breakpoints, as reported in Table 5, were derived from the published body of research and from national and European monitoring procedures. The antibiotics listed: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, quinupristin+dalfopristin, tetracycline and chloramphenicol were chosen to maximise the identification of resistance genotypes by assessing the resistance phenotypes.

In Gram-positive, bacteria acquired trimethoprim resistance, although occasionally detected is relatively rare. The data available (Korhonen et al., 2007) indicate that within species of lactobacilli the range of apparent trimethoprim resistances can be wide with no clear breakpoint values. Therefore, the MIC testing of trimethoprim for LAB was not considered relevant. Furthermore, testing for linezolid and neomycin is no longer considered necessary. The extremely rare non-mutational resistance to linezolid is due to the acquisition of the *cfz* gene, which also confers resistance to chloramphenicol (Arias et al., 2008; Toh et al., 2007). Testing for chloramphenicol resistance will efficiently cover for the hazard of acquiring resistance to linezolid. Neomycin is removed from the list since testing for the remaining three aminoglycosides efficiently covers the hazard of acquiring resistance to aminoglycosides.

Antibiotic susceptibility testing may be performed using different phenotypic test methods. In Clinical and Laboratory Standards Institute (CLSI), formerly National Committee on Clinical Laboratory Standards (NCCLS), the approved standards state that the methods of choice are agar dilution and broth microdilution (Anonym, 2007). Other widely used methods include the agar gradient method and commercial methods, such as Etest, which consists of a predefined gradient of antibiotic concentrations on a plastic strip (AbBiomérieux, Sweden). In addition to phenotypic antibiotic resistance determinations, also genotypic detection of particular genes causing resistance may be performed. These genotypic methods include different PCR -based methods, southern hybridization, plasmid profiling and microarray (Ammor et al., 2008; Aquilanti et al., 2007). The situation is clearest

Lactic acid bacteria	ampicillin	vancomycin	gentamicin**	Kanamycin**	streptomycin**	erythromycin	clindamycin	quinupristin+ dalphopristin	tetracycline	chloramphenicol
<i>Lactobacillus obligate homofermentative</i>	1	2	16	16	16	1	1	4	4	4
<i>Lactobacillus helveticus</i>	1	2	16	16	16	1	1	4	4	4
<i>Lactobacillus acidophilus</i> group	1	2	16	16	16	1	1	4	4	4
<i>Lactobacillus delbrueckii</i>	1	2	16	16	16	1	1	4	4	4
<i>Lactobacillus obligate heterofermentative</i>	2	n.r.	16	16	64	1	1	4	8	4
<i>Lactobacillus reuteri</i>	2	n.r.	8	16	64	1	1	4	16	4
<i>Lactobacillus fermentum</i>	1	n.r.	16	32	64	1	1	4	8	4
<i>Lactobacillus facultative heterofermentative*</i>	4	n.r.	16	64	64	1	1	4	8	4
<i>Lactobacillus plantarum</i>	2	n.r.	16	64	n.r.	1	1	4	32	8
<i>Lactobacillus rhamnosus</i>	4	n.r.	16	64	32	1	1	4	8	4
<i>Lactobacillus paracasei</i>	2	n.r.	32	64	n.r.	1	1	4	4	4
<i>Bifidobacterium</i>	2	2	64	n.r.	128	0.5	0.25	1	8	4
<i>Enterococcus</i>	4	4	32	512	128	4	4	4	2	8
<i>Pediococcus</i>	4	n.r.	16	64	64	1	1	4	8	4
<i>Leuconostoc</i>	2	n.r.	16	16	64	1	1	4	8	4
<i>Lactococcus lactis</i>	2	4	32	64	64	2	4	4	4	8
<i>Streptococcus thermophilus</i>	2	4	32	64	64	2	2	4	4	4
<i>Bacillus</i> spp.	n.r.	4	4	8	8	4	4	4	8	8
<i>Propionibacterium</i>	2	4	64	64	64	0.5	0.25	0.5	2	2
Other Gram (-)	1	2	4	16	8	0.5	0.25	0.5	2	2

n.r. not required;

\*including *Lactobacillus salivarius*;

\*\*possible interference of the growth medium

Table 5. Microbiological breakpoints categorizing bacteria as resistant (mg L<sup>-1</sup>). Strains with MIC higher than the breakpoints below are considered as resistant.

when the phenotypic and genotypic resistance patterns are in agreement. However, a phenotypically resistant strain may be genotypically “susceptible”. This is usually due to the fact that appropriate genes are not included in the test patterns, or there might be unknown resistance genes. Tetracycline, for example, has more than 40 different genes conferring antibiotic resistance discovered at the moment, and the number of tetracycline resistance genes continues to increase (Roberts, 2005). In contrast, a susceptible phenotype may also carry silent genes, which are observed with genotyping.

However, there is still a lack of agreement on the resistance-susceptibility breakpoints for most antibiotics in LAB (Charteris et al., 1998; Danielsen & Wind, 2003; Katla et al., 2001). Generally, the choice of medium has been shown to have a profound impact on the MICs of

LAB. The recommended growth media by the National Committee for Clinical Laboratory Standards (Mueller-Hinton agar) (NCCLS, 2002) and by the British Society for Antimicrobial Chemotherapy (Iso-Sensitest agar) (Andrews, 2001) do not support growth of all LAB. MRS medium, that generally supports the growth of LAB much better, is not always compatible to the Iso-Sensitest medium for the use in susceptibility testing, as was reported for various classes of antibiotics (Huys et al., 2002). Furthermore, there are still no guidelines available for the interpretation of susceptibility test results of commensal or food-associated bacteria. Additionally, MIC breakpoints values have been shown to be species specific and thus vary between species of the same genera (Danielsen & Wind, 2003). Also, distinguishing between intrinsic, non-specific and acquired resistance is difficult and requires, besides the evaluation of genetic base of resistance, that the antimicrobial-resistance patterns of many LAB species from different sources may be compared (Teuber et al., 1999).

## 5.2 Mobile genetic elements in LAB

A prerequisite for LAB to acquire antibiotic resistance genes from other bacteria is their ability to communicate actively and passively with these bacteria with the aid of conjugative plasmids and transposons. Conjugative plasmids and transposons are common in LAB, and due to their wide environmental distribution, it is possible that these commensal bacteria act as vectors for the dissemination of antibiotic resistance determinants to the consumer via the food chain. Plasmids are found in many genera of LAB, characterized by different size, function and distribution (Davidson et al., 1996; Wang & Lee, 1997). The functions related to the plasmids include hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins and exopolysaccharides, and resistance to antibiotics, heavy metals and phages. At least 25 species of lactobacilli contain native plasmids (Wang & Lee, 1997), and often appear to contain multiple (from 1 to 16) different plasmids in a single strain. R-plasmids encoding tetracycline, erythromycin, chloramphenicol, or macrolide-lincomycin-streptogramin resistance have been reported in *L. reuteri* (Lin et al., 1996; Tannock et al., 1994), *L. fermentum* (Fons et al., 1997; Ishiwa & Iwata, 1980), *L. acidophilus* (Vescovo et al., 1982), and *L. plantarum* (Danielsen, 2002) isolated from raw meat, silage and faeces. The reported prevalence of antibiotic resistance genes such as erythromycin, vancomycin, tetracycline, chloramphenicol, and gentamicin resistance genes, on transferable genetic elements in enterococci is more extensive, both on plasmids (Murray et al., 1988) and transposons (Clewell et al., 1995; Perreten et al., 1997a; Rice & Marshall, 1994;). A multiple antibiotic resistance plasmid was reported in a *L. lactis* strain isolated from cheese (Perreten et al., 1997b), encoding streptomycin, tetracycline and chloramphenicol resistance.

Conjugative transposons are the major vehicle regarding antibiotic resistance transport in LAB. They have been discovered in *E. faecalis* (Tn916, Tn918, Tn920, Tn925, Tn2702), *E. faecium* (Tn5233) and *L. lactis* (Tn5276, Tn5301). In enterococci and streptococci, resistances to tetracycline (tet (M)), erythromycin (ermAM, erm), chloramphenicol (cat) and kanamycin (aphA-3) have been determined. In lactococci, code for nisin (nis) production and sucrose fermentation (sac) has been observed. These transposons vary in size between 16 and 70 kb and may be inserted into plasmids or the chromosome in one or multiple copies. They may mobilize plasmids or chromosomal genes. The most remarkable observation is the extreme host range, which is the property of the Tn916/Tn1545 family.

### 5.3 Horizontal transferability of antibiotic resistance from LAB in food chain

The possible transfer of antibiotic resistance genes between bacterial species have been studied mostly in harmful or pathogenic species, but also recently in LAB. The vast majority of the experiments have been made *in vitro*, using methods such as filter-mating (Klare et al., 2007, Ouoba et al., 2008), although these *in vitro* methods do not mimic the circumstances in nature, and results obtained cannot be compared with the results achieved or expected using *in vivo* methods. The transferability of antibiotic resistance genes in the GI tract from LAB is not straightforward, since the GI tract is a hostile environment to many allochthonous bacteria. Moreover, studies made *in vivo* usually are based on "worst-case scenario", simulating very high daily intake of food products containing the resistant bacteria (Jacobsen et al., 2007). The potentially transferable genes in LAB have been described in multiple studies and have been reviewed in Ammor et al. (2007). Two of the most commonly observed resistance genes in LAB found so far are *tet(M)* for tetracycline resistance and *erm(B)* for erythromycin, followed with *cat* genes coding for chloramphenicol resistance (Cataloluk & Gogebakan 2004; Danielsen, 2002).

Enterococci are known to be very well receptive for conjugation (Clewell & Weaver, 1989), but are also successful donor organisms for the transfer of antibiotic resistance genes to unrelated enterococci (Rice et al., 1998), lactobacilli (Shrago & Dobrogosz, 1988), other Gram-positives including *Bacillus subtilis* (Christie et al., 1987), *Staphylococcus* (Young et al., 1987) and *Listeria* spp. (Charpentier et al., 1997; Perreten et al., 1997b), and even Gram-negative bacteria (Courvalin, 1994). Moreover, the transfer of conjugative elements, including a plasmid-encoded kanamycin resistance and a transposon-encoded tetracycline and erythromycin resistance (Doucet-Populaire et al., 1991), were shown to be transferable from *E. faecalis* to *Escherichia coli* and *Listeria monocytogenes*, respectively, in the digestive tract of mice. In contrast, reports of conjugative transfer of antibiotic resistance genes in other LAB are rare. Two *in vivo* studies were performed, to examine the possibility of conjugative transfer between native Gram-positive members of the gut. Therefore, the broad host range conjugative plasmid pAM $\beta$ 1 was transferred *in vitro* to *L. reuteri* (Morelli et al., 1988) and *L. lactis* (Igimi et al., 1996) and administered orally or using gastric intubation to mice. By analysis of faecal content, plasmid transfer to *E. faecalis* was observed in both studies.

In order to fully understand the extent to which LAB strains transfer resistance genes in the natural environment, it is essential to study genetic exchange in this context. Toomey et al., (2009) reported on the ability of wild-type antibiotic resistance determinants [*erm(B)* and *tet(M)*], present in LAB strains isolated from food sources, to be transferred to recipient strains. *In vitro* mating, using a traditional filter mating technique, showed that all four LAB mating pairs transferred their resistance determinants at high frequencies. By employing two *in vivo* models, an alfalfa sprout plant and an animal rumen model Toomey et al., (2009) demonstrated the transfer of resistance determinants between all four LAB mating pairs in these models. Previously, *in vivo* transfer between LAB has only been shown in the gastrointestinal tracts of gnotobiotic rats (Jacobsen et al., 2007) and mice (McConnell et al., 1991; Morelli et al., 1988). The transfer frequencies have been observed to increase when the animals have received the antibiotic in question at subtherapeutic levels (Igimi et al., 1996; Licht et al., 2003; Salyers & Shoemaker 1996) in their drinking water or feed, suggesting that increasing the antibiotic pressure can amplify the transfer of antibiotic resistance between

bacterial species. All of these above studies indicate that antibiotic resistant factors may be transferred from food related bacterium species (LAB) to other, potentially pathogenic species. The risks associated need to be considered, in light of the increasing concerns related to food as a potential reservoir for antibiotic resistance determinants.

## 6. Antibiotic resistance/susceptibility patterns of specific LAB genera applicable as probiotics

Some features appeared to be shared by the majority of LAB; in particular, it was reported that most LAB species are resistant to metronidazole and that they are all intrinsically resistant to sulphonamides and trimethoprim, while they are usually susceptible to piperacillin and piperacillin plus tazobactam. On the other hand, clear differences were highlighted among different LAB genera, although well-defined species-specific profiles were not always identifiable. A high resistance to ceftiofur was acknowledged for *Lactococcus*, *Leuconostoc*, and *Lactobacillus*, whereas, as regards vancomycin, *Leuconostoc*, *Pediococcus* and most lactobacilli species were recognised as intrinsically resistant and most *Lactococcus* isolates as highly susceptible.

Lactobacilli widely used in starter cultures or as probiotics in dairy products enter human intestines in large numbers and there interact with the intestinal microbiota (Teuber et al., 1999). Therefore they have the potential to serve as hosts for antibiotic-resistance genes, with the risk of transferring the genes to opportunistic or pathogenic bacteria. Routine antibiotic susceptibility testing has been advocated as an essential selection criterion for potentially starter or probiotic *Lactobacillus* cultures (Charteris & Kelly, 1993). The *Lactobacillus* species have been found susceptible to many cell wall synthesis inhibitors, like penicillins and ampicillin (Danielsen & Wind 2003, Coppola et al., 2005), in contrast to glycopeptides such as vancomycin, most *Lactobacillus* species, excluding obligate heterofermentative species, have been found to be resistant to these types of antibiotics. However, the resistance towards vancomycin has been demonstrated being as intrinsic (Tynkkynen et al., 1998) due the presence of D-alanine: D-alanine ligase-related enzymes (Elisha & Courvalin, 1995) and should not be compared with transmissible, plasmid-mediated resistance found in enterococci (Leclercq et al., 1992). As a general rule, lactobacilli have a high natural resistance to bacitracin, ceftiofur, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin (Danielsen & Wind, 2003). In addition, resistance against inhibitors of nucleic acid synthesis, such as trimethoprim, seems to be intrinsic, although further characterizations are required on this topic (Ammor et al., 2007). Resistance to tetracycline has been observed more often among *Lactobacillus* species, and it has been shown to have a wide range of MICs (Korhonen et al., 2008), also with a multimodal distribution of MICs, probably due to the extensive variability of tetracycline resistance mechanisms conferring diverse levels of susceptibility (Roberts, 2005). Especially with tetracycline, molecular methods should be applied in order to reveal the nature of resistance, i.e. is it due to intrinsic mechanisms, mutation or added, mobile genes.

Screening of antibiotic-resistance profile among *Lactobacillus* strains used in dairy products such as probiotics or as starters is now tending to become systematic. Coppola et al., (2005) pointed out that all of 63 *L. rhamnosus* strains isolated from Parmigiano Reggiano cheese showed resistance to six antibiotics (ceftiofur, vancomycin, neomycin, enoxacin, pefloxacin,

and sulphamethoxazole plus trimetoprim). Investigating the current antibiotic-resistance situation in microbial food additives in Switzerland, Kastner et al., (2006) determined that among 74 *Lactobacillus* isolates applicable as starter or probiotic cultures, two antibiotic resistances were detected in probiotic cultures. The genetic base of those resistances was confirmed; the tetracycline resistance gene tet(W) in *L. reuteri* SD 2112 (residing on a plasmid) and the lincosamide resistance gene lnu(A) in *L. reuteri* SD 2112. The similar trend was noticed in study of Katla et al., (2001). Only one of the 189 *Lactobacillus* strains isolated from Norwegian dairy products such as yoghurt, sour cream, fermented milk and cheese was classified as high level resistant to streptomycin. In contrast, a study conducted on "home-made" spanish cheese (Serena, Gamoneda, Cabrales) revealed the presence of lactobacilli resistant to penicillin G, cloxacillin, streptomycin, gentamycin, tetracycline, erythromycin and chloramphenicol (Herrero et al., 1996).

*L. lactis* strains were sensitive to amikacin, ampicillin, 1st generation cephalosporin, chloramphenicol, erythromycin, gentamicin, imipenem, oxacillin, penicillin, piperacillin, sulphonamide, tetracycline, trimethoprim/sulfomethoxazole, and vancomycin (de Fabrizio et al., 1994). A slightly lowered susceptibility was observed towards carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin. Intrinsic resistances were recorded towards colistin, fosfomycin, pipemidic acid and rifamycin. Orberg & Sandine (1985) demonstrated that investigated strains of *L. lactis* subsp. *cremoris* and subsp. *lactis* were all resistant to thrimethoprim and almost all to sulphathiazole. Resistance to gentamicin, kanamycin, lincomycin, neomycin, rifampin and streptomycin varied.

The enterococcal strains are naturally tolerant to  $\beta$ -lactams, cephalosporins, lincosamides and polymyxins. A specific cause for concern and a factor contributing to the pathogenesis of enterococci is the resistance they acquire to aminoglycosides, tetracyclines, macrolides, chloramphenicol, penicillin, and ampicillin (Gray et al., 1991) and their capacity to exchange genetic information by conjugation. Enterococcal food isolates (mainly *E. faecalis* and *E. faecium*) were analysed for resistances to a broader range of different antibiotics using phenotypic susceptibility testing, both in raw meat (Knudtson & Hartman, 1993; Quednau et al., 1998) and fermented milk and meat products (Franz et al., 2001; Teuber & Perreten, 2000). Their data suggest a high prevalence of (multiple) antibiotic resistant enterococci in foods, which nevertheless were mostly susceptible to the clinically relevant antibiotics ampicillin and vancomycin. Enterococci from European cheeses, mainly belonging to *E. faecalis* and *E. faecium*, are susceptible to different antibiotics in different proportions (Teuber et al., 1999; Franz et al., 2001). From the study of European cheeses Teuber et al. (1999) ascertained that the incidence for vancomycin resistance among enterococcal isolates was as low as 4%. When Franz et al. (2001) tested 47 *E. faecalis* strains, isolated mostly from cheeses, they were all susceptible to vancomycin. Bulajić & Mijačević (2011) pointed out that among enterococcal strains isolated from autochthonous Sombor cheese, only one strain showed vancomycin resistance. In contrast, Citak et al. (2004) have shown resistance to vancomycin among the population of enterococci isolated from Turkish white cheeses and was found in 96.8% of *E. faecalis* isolates, and 76% of *E. faecium* strains. The susceptibility to vancomycin is of great importance as this glycopeptide antibiotic is one of the last therapeutic options in clinical therapy.

Bifidobacteria are generally considered to be food-grade organisms that do not impose health risks on the consumer or the environment. Nevertheless, it should be noted that rare

cases of *Bifidobacterium*-associated gastrointestinal and extra-intestinal infections have been described. In contrast to susceptibility testing of clinically important bacteria, no standard procedures are specifically dedicated to the determination of resistance phenotypes in *Bifidobacterium* strains. To date, a large variety of methods and protocols have been described for antimicrobial susceptibility testing of bifidobacteria, including agar (overlay) disc diffusion, broth dilution and agar dilution. In addition, various growth media have been used primarily on the basis that they meet the complex growth requirements of bifidobacteria. As opposed to conventional susceptibility test media such as Mueller–Hinton and Iso-Sensitest medium none of these *Bifidobacterium*-specific media are well defined in terms of minimal interaction between specific antimicrobial agents and growth medium components. Recently, a newly defined medium formulation referred to as the Lactic acid bacteria Susceptibility test Medium supplemented with cysteine (LSM + cysteine) was proposed for susceptibility testing of bifidobacteria.

Moubareck et al., (2005) were tested the fifty bifidobacterial strains, isolated from humans, animals or probiotic products for susceptibility to 30 antibiotics by disc diffusion test on *Brucella* agar supplemented with 5% laked sheep blood and vitamin K (1mg/L). All strains were sensitive to penicilins: penicillin G, amoxicillin, piperacillin, ticarcillin, imipenem, and usually anti-Gram-positive antibiotics (macrolides, clindamycin, vancomycin and teicoplanin). Most isolates (70%) were resistant to fusidic acid and, as expected, high resistance profile were observed for aminoglycosides. Potentially acquired resistance was only observed against tetracycline and minocycline, in 14% of the tested strains. For the first time, Moubareck et al., (2005) identified *tet(W)* as the gene responsible for tetracycline resistance in *Bifidobacterium pseudocatenulatum* and *B. bifidum*. Interestingly, the *tet(W)* gene was previously found in human *B. longum* and three genera of rumen obligate anaerobes, suggesting intergenic transfer of this resistance gene between anaerobic bacteria (Scott et al., 2000). In the study of Masco et al., (2006), the LSM + cysteine medium was used to determine the susceptibility profile of 100 bifidobacterial isolates (strains of animal and human origin, isolates from probiotic products and strains from clinical sources) to 15 common antimicrobial agents. All strains tested were susceptible to amoxicillin, chloramphenicol, erythromycin, quinupristin/dalfopristin, rifampicin and vancomycin. The data from this study (Masco et al., 2006) also reinforce earlier observations indicating that bifidobacteria are intrinsically resistant to gentamicin, sulfamethoxazole and polymyxin B. Susceptibility to trimethoprim, trimethoprim/sulfamethoxazole, ciprofloxacin, clindamycin, tetracycline and minocycline was variable. The *tet(W)* gene was responsible for tetracycline resistance in 15 strains including 7 probiotic isolates belonging to the taxa *Bifidobacterium animalis* subsp. *lactis* and *B. bifidum*. This gene was present in a single copy on the chromosome and did not appear to be associated with the conjugative transposon TnB1230 previously found in *tet(W)*-containing *Butyrivibrio fibrisolvens*.

## 7. Conclusion

The selective pressure imposed by the use of antimicrobial agents plays a key role in the emergence of resistant bacteria. Under selective pressure, the numbers of these bacteria increase and some may transmit their resistance genes to other members of the population.. The food chain was considered as the main route of transmission of antibiotic resistant lactic acid bacteria between the animals and human population. Fermented dairy products and

fermented meats, which are not heat-treated before consumption, provide a vehicle for antibiotic resistant LAB with a direct link between the animal indigenous microflora and the human gastrointestinal tract. There is the potential health risk, due to the transfer of antibiotic resistance genes from LAB to bacteria in the human gastrointestinal tract, especially to pathogenic bacteria.

Lactic acid bacteria used as starter cultures or probiotic bacteria, enter into human intestines in large number where they interact with the intestinal microflora. Since there has been a significant rise in the consumption of probiotic products, it is important that probiotics are well documented regarding antibiotic resistance profile. The ability to transfer antibiotic resistance genes must be considered as an important parameter for the selection of the probiotic strains. Continuous attention should be paid to the selection of probiotic strains free of transferable antibiotic-resistance determinants. Without doubt, the uncontrolled use of antimicrobial agents in farming practice has assisted the spread of resistant organisms. Therefore a much stricter control over the use of these drugs is essential.

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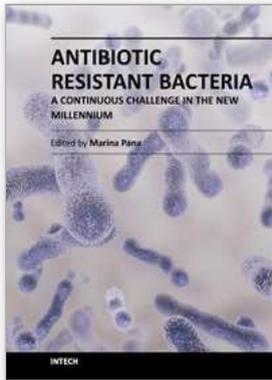
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Antibiotic-resistant bacterial strains remain a major global threat, despite the prevention, diagnosis and antibiotherapy, which have improved considerably. In this thematic issue, the scientists present their results of accomplished studies, in order to provide an updated overview of scientific information and also, to exchange views on new strategies for interventions in antibiotic-resistant bacterial strains cases and outbreaks. As a consequence, the recently developed techniques in this field will contribute to a considerable progress in medical research.

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