REVIEW
OF
LITERATURE
INTRODUCTION

Pasteur was the first to say that life of upper animals would be impaired by the absence of indigenous microorganisms but was his colleagues Metchnikoff who, exactly one century ago, to observe that: "the different susceptibilities of people to the harmful action of microbes and their products. Some can swallow without any evil result a quantity of microbes which in the case of other individuals would produce a fatal attack of cholera. Everything depends upon the resistance offered to the microbes by the invaded organism." (Metchnikoff 1907)

He also stated that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes." (Metchnikoff 1907)

This sentence describes in a clear way the "probiotic concept", the use of health promoting bacteria able to exert a positive role on intestinal flora, even if the word probiotics was coined several dozen years later; then we can assume today we are going to celebrate the first century of life of probiotics.

There is a tendency to regard all micro organisms as harmful; to equate bacteria with germs. Nothing could be further from the truth. The number of non pathogenic species far exceeds the number of pathogenic species and many of the non pathogens are in fact useful, even essential for the continued existence of life on earth.

Inside each of us live vast numbers of bacteria without which we could not remain in good health. There are several thousand billions in each person (more than all cells in the body) divided into four hundred species, most of them living in the digestive tract.

PROBIOTICS

Probiotics are defined as viable microbial dietary supplements that, when introduced in sufficient quantities, beneficially affect human organism through their effects in the intestinal tract. (Dimer & Gibson 1998; Zimmer & Gibson 1998; Sanders 1998; Vaughan et al., 1999; Zubillaga et al., 2001; Holzapfel & Schillinger 2002)
The physiological effects related to probiotic bacteria include the reduction of gut pH, production of some digestive enzymes and vitamins, production of antibacterial substances, e.g., organic acids, bacteriocins, hydrogen peroxide, diacetyl, acetaldehyde, lactoperoxidase system, lactones and other unidentified substances, reconstruction of normal intestinal microflora after disorders caused by diarrhoeas, antibiotic therapy and radiotherapy, reduction of cholesterol level in the blood, stimulation of immune functions, suppression of bacterial infections, removal of carcinogens, improvement of calcium absorption as well as the reduction of faecal enzyme activity. (Ouwehand et al., 1999; Zubillaga et al., 2001; Holzapfel & Schilling 2002)

The probiotic microorganisms should not be pathogenic, have no connection with diarrhoeagenic bacteria and no ability to transfer antibiotic resistance genes, as well as be able to maintain genetic stability. To be recognized as functional food components, they should demonstrate the following properties: acid- and bile-stability, resistance to digestive enzymes, adhesion to intestine surface, antagonistic activity against human pathogens, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects, stimulation of the immune system without inflammatory effects, enhancement of bowel motility, maintenance of mucosal integrity, improvement of bioavailability of food compounds and production of vitamins and enzymes. (Ouwehand et al., 1999) The technological properties of bacteria play a very significant role in the production of probiotics. (Saarela et al., 2000)

They possess good sensorial properties, fermentative activity, good survival during freeze-drying or spray-drying, proper growth and viability in food products, phage resistance and high stability during long-term storage. The majority of bacteria belonging to the Lactobacillus and Bifidobacterium genera are recognized as safety. It is generally accepted that, with the only exception of streptococci and enterococci, lactic acid bacteria are rarely pathogenic to humans and animals. They have been used in production of foods since ancient times with no negative effects on humans. However, the list of probiotic strains is rather short. It includes strains offered by the dairy industry and some scientific groups. (Ouwehand et al., 1999; Holzapfel & Schillinger 2002)
MECHANISM OF PROBIOTICS

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained. (Holzapfel et al., 1998) There are studies on how probiotics work. So, many mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders. These mechanisms listed below briefly. (Rolfe 2000, Čakır 2003, Salminen et al., 1999, Castagliuola et al., 1999)

1. Production of inhibitory substances: Production of some organic acids, hydrogen peroxide and bacteriocins which are inhibitory to both gram-positive and gram-negative bacteria.
2. Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition. Probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.
3. Competition for nutrients: Despite of the lack of studies in vivo, probiotics inhibit the pathogens by consuming the nutrients which pathogens need.
4. Stimulating of immunity: Stimulation of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjuvants and increase humoral immune response.
5. Degradation of toxin receptor: Because of the degredation of toxin receptor on the intestinal mucosa, it was shown that S. boulardii protects the host against C.difficile intestinal disease.

Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence. (Fooks et al., 1999)

PREBIOTICS

Prebiotics are an alternative for probiotics or their cofactors. They are defined as nondigestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of one or a limited number of probiotic bacteria in the colon. (Crittenden & Playne 1996; Dimer & Gibson 1998; Zimmer & Gibson 1998; Manning & Gibson 2004)

Prebiotic oligosaccharides can be produced in three different ways: by extraction from plant materials, microbiological synthesis or enzymatic synthesis, and enzymatic hydrolysis of polysaccharides. (Crittenden & Playne 1996; Gulewicz et al., 2003) The majority of
prebiotic oligosaccharides are produced on the industrial scale and are widely available on the market. Recently, many patents concerning probiotic oligosaccharides have been claimed and this field is continuously increasing. (Crittenden & Playne 1996)

Free radicals are a major cause of many degenerative diseases, such as atherosclerosis, cancer, cardiovascular diseases, inflammatory bowel diseases, skin aging, old age dementia diseases. (Shklar 1998; Surh 1999; Kris-Etherton et al., 2002; Ferrari & Torres 2003)

Epidemiological data and randomized clinical trials provide ample indications that antioxidants play a fundamental role in the prevention of cancer and cardiovascular reactive oxygen species and metal chelators that protect human cells and reduce oxidative damages.

The Russian scientist and Nobel laureate Eli Metchnikoff first introduced the positive role of certain bacteria to the human body. In the beginning of the 20th century he suggested that it would be possible to replace harmful microbes with useful ones. At that time he was a professor at the Pasteur Institute in Paris (Metchnikoff E. 1907) He believed that the aging process was due to toxins such as phenols, indols and ammonia in the large intestine, produced by proteolytic microbes such as clostridia. Clostridia are normal to the gut. He noted that milk fermented with lactic acid bacteria inhibited the growth of the proteolytic bacteria because of the low pH produced by the fermentation of the lactose. Metchnikoff also observed that certain rural peoples in Europe such as in Bulgaria, who lived mainly on milk fermented with lactic acid bacteria, lived a relatively long life. He then introduced sour milk fermented with the bacteria to his diet and found his health benefited from the consumption. He called it “Bulgarian Bacillus”.

The first person to isolate Bifidobacterium was Henry Tissier. He also was from the Pasteur Institute. He isolated the bacterium from a breast-fed infant and called it Bacillus bifidus communis. It was later renamed Bifidobacterium bifidum. Tissier concluded it was the predominante microflora in breast-fed infants and recommended it for babies suffering from diarrhea. (Tissier H. 1900)

In 1917, the German professor Alfred Nissle isolated the bacterium Escherichia coli from the faeces of a World War I soldier who didn’t develop enterocolitis when he had a severe case of shigellosis. He used the strain to treat intestinal diseases such as shigello...
salmonellosis with a considerable amount of success. At that time antibiotics weren’t yet discovered. The probiotic *Escherichia coli* are still in use today. It’s been shown to directly interact with the adaptive immune system. *(Kruis W. 2004-Nissle 1917)*

**MEDIA FOR ISOLATION**

Often abbreviated to MRS, this type of bacterial growth medium is so-named by its inventors: de Man, Rogosa and Sharpe. Developed in 1960, this medium was designed to favour the luxuriant growth of *Lactobacilli* for lab study. It contains sodium acetate, which suppresses the growth of many competing bacteria (although some other Lactobacillales, like *Leuconostoc* and *Pediococcus*, may grow). This medium has a clear brown colour. *(EMD Chemicals, MRS Agar 2002)*

MRS agar typically contains (w/v):

- 1.0 % peptone
- 0.8 % meat extract
- 0.4 % yeast extract
- 2.0 % glucose
- 0.5 % sodium acetate trihydrate
- 0.1 % polysorbate 80 (also known as Tween 80)
- 0.2 % dipotassium hydrogen phosphate
- 0.2 % trimmonium citrate
- 0.02 % magnesium sulfate heptahydrate
- 0.005 % manganese sulfate tetrahydrate
- 1.0 % agar
- pH adjusted to 6.2 at 25°C

The yeast and meat extracts and peptone provide sources of carbon, nitrogen and vitamins for general bacterial growth. The yeast extract also contains vitamins and amino acids specifically required by *Lactobacilli*. polysorbate 80 is a surfactant which assists in nutrient uptake by *Lactobacilli*. Magnesium sulfate and manganese sulfate provide cations used in metabolism. *(de Man et al., 1960)*
ISOLATION

At the start of the 20th century, probiotics were thought to beneficially affect the host by improving its intestinal microbial balance, thus inhibiting pathogens and toxin producing bacteria. (Metchnikoff E. 1907) Today, specific health effects are being investigated and documented including alleviation of chronic intestinal inflammatory diseases (Mach. T. 2006), prevention and treatment of pathogen-induced diarrhoea urogenital infections (Yan F, Polk DB. 2006), and atopic diseases. (Vanderhoof JA 2008)

Lactic acid bacteria (LAB) are Gram-positive, non--spore forming, catalase-negative bacteria that are devoid of cytochromes and are of nonaerobic habit but are aero-tolerant, fastidious, acid tolerant and strictly fermentative; lactic acid is the major end-product of sugar fermentation. They are the most widely used bacteria as starter cultures for the industrial processing of fermented dairy, meat, vegetable and cereal products. Reduction of pH and conversion of sugars to organic acids are the primary preserving actions that these bacteria provide to fermented food. (L.T. Axelsson 1989)

The isolated bacteria were identified as Lactobacillus spp. by observing their morphological characteristics and by means Gram staining, motility test, catalase test, endospore test, milk coagulation activities. (M.Z. Hoque et al., 2010)

Lactobacilli are an extremely important group of probiotic bacteria inhibit undesirable microflora in the gut and create a healthy equilibrium between beneficial and potentially intestinal pathogens. The study was attempted to isolate Lactobacilli strains from goat milk to search for a new effective antibacterial probiotic strains. From 40 raw goat milk samples, 48 Lactobacilli isolates were isolated, identified and analyzed for their probiotic properties including acid and bile salt tolerance, antibacterial activity against enteric pathogens, antibiotic resistance patterns and production of bacteriocin. Out of these Lactobacillus species, five isolates were potential probiotics, and bacteriocins produced by them showed demonstrable antibacterial activity against the test pathogens such as S. typhi, Pr. vulgaris, K. pneumoniae, Sh. flexneri, E. aerogenes and E. coli. The bacteriocin produced by L. plantarum (G95a) and L. rhamnosus (G119b) was prominent antibacterial, resistant to heat at 1210C and tolerated acidic pH 3 but sensitive to pH 9. The present study suggested that
isolated Lactobacillus strains have an excellent probiotic potential and control of enteric infections and restoration of microbial gut flora. (D.H. Tambekar and S.A. Bhutada 2010)

**BIOCHEMICAL CHARACTERIZATION**

Biochemical characterization is primary aspect of identification of the culture or the isolates obtained.

Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. (Chelikani P. et al., 2004)

Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules. Probiotics isolates as per the Bergey’s manual catalase negative, which is the significant character of probiotics. (Gaetani G. et al., 1996)

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to split indole from the amino acid tryptophan. This division is performed by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase." (Macfaddin 1980) As per the Bergey’s manual indole production test is negative of probiotics isolates.

This test is used to determine two things. The MR portion (methyl red) is used to determine if glucose can be converted to acidic products like lactate, acetate, and formate. The VP portion is used to determine if glucose can be converted to acetoin. As per the bergey’s manual MR test is positive and VP test is negative of probiotics isolates.

Methyl red is an indicator dye that turns red in acidic solutions. It is an azo dye, and is a dark red crystalline powder. Methyl red is a pH indicator; it is red in pH under 4.4, yellow in pH over 6.2, and orange in between, with a pKa of 5.1 (H. T. Clarke and W. R. Kirner 1941)
Tests for the ability of bacteria to convert citrate (an intermediate of the Kreb’s cycle) into oxaloacetate (another intermediate of the Kreb’s cycle). In this media, citrate is the only carbon source available to the bacteria. If it cannot use citrate then it will not grow. If it can use citrate, then the bacteria will grow and the media will turn a bright blue as a result of an increase in the pH of the media. Probiotics isolates were not able to utilize citrate as according to bergey’s manual.

Gelatin agar media is used to test if bacteria can digest the protein gelatin. To digest gelatin, the bacteria must make an enzyme called gelatinase. Gelatin as a solidifying agent and source of protein, it is used as gelatine agar to study its utilization as well as for its liquification. According to bergey’s manual any species of probiotics are not able to utilize gelatin and not able to liquefy it.

In starch hydrolysis, undegraded starch reacts with Iodine to form a dark blue starch-iodine complex that covers the entire agar. If starch (polysaccharide) is broken down into glucose (or any other monosaccharides/ di saccharides), glucose will then react with iodine, forming a clear zone surrounding streak line.

As one of the best source of carbohydrate starch is utilized or not can be studied by application of iodine.

Casein (a protein) is broken down by protease into peptones and amino acids. During the degradation process, polypeptide bonds are broken. Once the bonds are broken, amino acids are produced. A clear zone surrounding streak line of agar indicates positive result.

Decomposition of amino acid “cysteine” is detected by the formation of ferrous sulphide when Hydrogen Sulphide is release. When H2S is produced, sulphide ion reacts with the metal salt to produce a black precipitate positive result.

Urease breaks down urea into ammonia (NH3) & carbon dioxide (CO2). Phenol red is used as pH indicator, pH indicator changes from yellow to bright pink if NH3 is produced.

Bacteria produce acidic products when they ferment certain carbohydrates. The carbohydrate utilization tests are designed to detect the change in pH which would occur if fermentation of the given carbohydrate occurred. Acids lower the pH of the medium which
will cause the pH indicator (phenol red) to turn yellow. If the bacteria do not ferment the carbohydrate then the media remains red. If gas is produced as a by product of fermentation, then the Durham tube will have a bubble in it. This is known as Sugar fermentation test.

Litmus milk has several components that can be metabolized: lactose (milk sugar); casein (milk protein); and litmus (a pH indicator that is purple to blue at neutral to alkaline pH and pink under acid conditions). If lactose is fermented, the solution should turn pink. If gas is produced during fermentation, bubble or cracks in the milky medium can be observed (but this is often difficult to observe). If lactose is not fermented and proteins are instead used for energy, the solution will become alkaline and more blue. Casein protein may be digested. This will coagulate the milk to form a curd (a solid) (Bergey’s manual).

**pH AND BILE SALT TOLERANCE**

Bacteria used as probiotic strains are joined in the food system with a journey to the lower intestinal tract via the mouth. In this food system, probiotic bacteria should be resistant to the enzymes like lysozyme in the oral cavity. Then the journey will be going on in the stomach and enter the upper intestinal tract which contain bile. In this stage strains should have the ability to resist the digestion processes. It is reported that time at the first entrance to release from the stomach takes three hours. Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile. (Chou and Weimer 1999, Çakır 2003)

To show probiotic sufficiencies, they should reach to the lower intestinal tract and maintain themselves over there. Because of desirable point the first criteria is looking for probiotic strains is being resistant to acid and bile. Bile acids are synthesized in the liver from cholesterol and sent to the gall –bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine this acids suffer some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity. Conjugated and deconjugated bile acids show antimicrobial activity especially on *E. coli* subspecies, *Klebsiella* spp., and *Enterococcus* spp. in vitro. The deconjugated acid forms are more effective on gram positive bacteria. (Dunne et al., 1999, Çakır 2003)
Among two of selected bifidobacterium strains, HJ 30 and SI 31, showed higher rates of survival. *(Chung et al., 1998)* In another study a large culture collection of lactic acid bacteria of NZDRI was screened to select strains to use as probiotics. For this, over 200 strains of *Lactobacillus* and *Bifidobacterium* were examined according to their ability of resistant to bile and acid and four of them selected. Three of them were from dairy origins and the last one was from human origin. They were compared with the two commercial probiotic strains namely *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* LA-1. The isolated strains were analyzed for a series of pH between 1 and 3 and also for tolerance against bile at final concentrations of 0, 0.5 and 1% w/v. They were tolerant for the conditions mentioned above. While the general survival patterns are similar, the strain from human orgin showed higher tolerance. These strains were identified as *Lactobacillus rhamnosus* HN001, *Lactobacillus rhamnosus* HN007, *Lactobacillus acidophilus* HN017 and *Bifidobacterium lactis* HN019. *(Prasad et al., 1998)*

In another research, twenty nine Lactobacillus strains of dairy origin were tested in vitro for their probiotic potential. The resistance of bacteria was examined in pH 1 between pH 3. Tolerance to bile salt was tested against to 0.3% oxgall. All of the examined strains were resistant to pH 3 during 3h, but most of them lost their viability in 1h in pH 1. Also all of them were tolerated 0.3% bile salts concentration in 4 h. For in vivo testing the most suitable strains were chosen, *L.casei* Shirota ACA-DC 6002, *L.plantarum* ACA-DC 146, *L.paracasei* subsp. tolerans ACA-DC 4037. *(Maragkoudakis et al. 2005)* Also an experiment was performed on three *Lactobacillus* species isolated from human milk whether they may use potential probiotic strains.

They were identified as *Lactobacillus gasseri* and one of them *Lactobacillus fermentum*. Survival in low pH and in gastrointestinal environment was examined for a comparison with commercial probiotic strains, *L.rhamnosus* GG, *L. casei* imunitass and *L. johnsanii* La1. The strains especially *L.gasseri* showed that it can be used as a potential probiotic strain. *(Martín et al. 2004)*

Bile is a yellow-green aqueous solution whose major constituents include bile acids, cholesterol, phospholipids, and the pigment biliverdin. It is synthesized in the pericentral
hepatocytes of the liver, stored and concentrated in the gallbladder interdigestively, and released into the duodenum after food intake. Bile functions as a biological detergent that emulsifies and solubilizes lipids, thereby playing an essential role in fat digestion. This detergent property of bile also confers potent antimicrobial activity, primarily through the dissolution of bacterial membranes. (M. Begley 2009, C. G. M. Gahan and C. Hill 2005)

Bile Salt Hydrolase activity has been detected in *Lactobacillus*, *Bifidobacterium* *Enterococcus* *Clostridium*, and *Bacteroides* spp. Lactobacilli and bifidobacteria are routinely used as probiotic strains, while *Bacteroides*, *Clostridium*, and *Enterococcus* spp. are also commensal inhabitants of the gastrointestinal tract. To date, BSH activity has not been detected in bacteria isolated from environments from which bile salts are absent (e.g., *Lactococcus lactis* or *Streptococcus thermophilus*). With the exception of two strains of *Bacteroides*, all other BSH-positive bacteria are gram positive. All other gram-negative intestinal bacteria that have been examined (including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium) neither demonstrate BSH activity nor possess *bsh* homologs in their genome. (Holzapfel W. H. and P. Haberer 1998)

*bsh* mutant pairs provide a link between bile salt hydrolysis and bile tolerance. A *Lactobacillus amylovorus* mutant with a partial decrease in BSH activity isolated using an *N*-methyl-*N*¹-nitro-*N*-nitrosoguanidine mutagenesis strategy displayed decreased growth rates in the presence of bile salts. (J. M. Antoine and F. Schneider 2000) Also, mutation of *bsh* in *L. plantarum* and *L. monocytogenes* renders cells significantly more sensitive to bile and bile salts. However, it has been proposed that since the protonated (nondissociated) form of bile salts may exhibit toxicity through intracellular acidification in a manner similar to organic acids, BSH-positive cells may protect themselves through the formation of the weaker unconjugated counterparts (Leer R. J. et al., 1993)

Bile salts usually only have slight affects (if any) on bacterial cells at every pH examined, glycoconjugated bile salts are extremely toxic at acidic pH and *bsh* mutants are significantly more inhibited than corresponding parent cells. (C. G. M. Gahan, and C. Hill. 2005) Thus, BSHs are particularly important in combating the toxic effects of glycoconjugated bile salts at low pH, and BSH activity may be of particular importance at the point where bile enters the duodenum and where acid reflux may occur from the stomach, or in localized microenvironments in the intestine when the pH is lowered dsby lactic acid
bacteria. The fact that BSHs have been shown to preferentially hydrolyze glycoconjugated bile salts, together with the observation that BSHs have slightly acidic pH optima (usually between pH 5 and 6) may serve to substantiate this theory. (Tuohy K. M., et al., 2003)

Probiotics potential of LAB is necessarily its ability to resist bile salts and acidic pH. (Lee and Salminen 1995) In this study, three isolated excellent probiotic, commercial probiotic and standard probiotic bacterial strains showed acid tolerance at pH 2 and bile salt tolerance at 2%. Before reaching the intestinal tract, probiotic bacteria must first succeed in transit through the stomach where the pH can be as low as 1.5 to 2. (Dunne et al., 2001) Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host. (Havenaar et al., 1992) This will help Lactobacilli to reach the small intestine and colon and contribute in balancing the intestinal microflora. (Tambekar and Bhutada 2010)

Method was used to determine bile tolerance of selected LAB. Before testing for bile tolerance, LAB strains were grown at 37°C for 24 hour in MRS broth without bile. One ml of the culture broth was poured onto MRS agar with bile salt concentrations of 2000, 3000 and 4000 ppm. Bacterial growth was determined after incubation at 37°C for 48 hour. The modified method of (Erkkila and Petaja 2000) was applied in the study.

Bile salt tolerance of the Lactobacillus strains was able to grow in MRS agar supplemented with 3000 ppm bile salt. In the human GI-tract, the mean bile salt concentration is believed to be 3000 ppm, which is considered as critical and high enough to screen for resistant strains. (Gilliland et al., 1977; Goldin and Gorbach 1992)

However, L. rhamnosus strains isolated from Parmigiano Reggiano cheese were able to survive at bile salt concentration of 10,000, 15,000 and 20,000 ppm after 48 hour of incubation at 37°C. (Succi et al., 2005) It has been reported that certain strains of Lactobacillus are able to reduce this detergent effect by their ability to hydrolyze bile salt by bile salt hydrolase enzyme (BSH). (Erkkila and Petaja 2000)

NaCl TOLERANCE

NaCl is an inhibitory substance when applied in high amount and thus may inhibit growth of certain types of bacteria. Lactobacillus spp. isolated from yoghurt. Yoghurt samples were collected from Grameen Dannone yoghurt, Bogra and Khulna Districts of
Bangladesh. The isolated lactobacilli from yoghurts were able to tolerate 1-9% NaCl. NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. The current result respectively showed that \textit{Lactobacillus} spp. isolated from yoghurts. On the other hand, they were able to survive and were able to tolerate 1-9% of NaCl and good growth was had partial multiplication abilities at pH 6.60. In per ml observed at 1% NaCl. (M.Z. Hoque et al., 2010)

**ANTIBIOTIC SUSCEPTIBILITY**

Routine antibiotic susceptibility testing of lactic acid bacteria (LAB) and \textit{bifidobacteria} may be advisable in a number of instances e.g. for checking the biosafety of potentially probiotic isolates. In fact, there is concern over the possible spread of antibiotic resistance determinants from bacteria used in probiotic products. However, there is still a lack of agreement on the MIC interpretative breakpoints mainly for \textit{Lactobacillus} spp.

\textit{Bifidobacteria} are common inhabitants in the human and animal intestines, and are generally associated with good intestinal health. They are increasingly used as probiotics and one target for their probiotic use is attenuation of ecological disturbances in the microbiota caused by antibiotic therapy. \textit{Bifidobacteria} are typically susceptible to majority of clinically relevant antibiotics such as penicillins, cephalosporins and macrolides, and bifidobacterial population is therefore vulnerable to changes during antibiotic administration. However, their susceptibility to tetracycline is variable. (Moubareck et al., 2005, Delgado et al., 2005)

Tetracycline group antibiotics are widely used for therapeutics and prophylaxis of clinical infections in humans and animals and in some countries they are additionally used as growth promoters in animals. Tetracycline resistance is increasingly common in bacteria living in a variety of ecological niches and several transferable determinants have been linked to the resistance. (Roberts, 1979) Ribosomal protection protein type resistance genes, especially \textit{tet} (W), have been detected in several \textit{Bifidobacterium} species. (Moubareck et al., 2005, Aires, 2007) However, the data on the species distribution of \textit{bifidobacterial} population and changes in antibiotic susceptibility patterns caused by tetracycline administration is scarce.
GROWTH CURVE

The first stage of the researches is represented by the determination, under laboratory conditions, of the evolution of *Lactobacillus plantarum* BS1 and BS3 strains in the case of using MRS medium. From the data presented it results that the two strains have a similar profile of the growth curve. The lag phase is very short and the exponential growth phase lasts for approximately 20 hours. The duplication time is shorter for *Lactobacillus plantarum* BS1. Instead, for *Lactobacillus plantarum* BS3 strain it results a greater productivity. The differences between the two strains can be attributed to the calculation and determination differences of the parameters considered. (E. Vamanu. et al., 2011)

ANTIMICROBIAL ACTIVITY

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial activity targets the enteric undesirables and pathogens. (Klaenhammer Kullen 1999) Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins. (Quwehand and Vesterlund 2004, Çakır 2003) Till today there are some researches on showing that different species produce different antimicrobial substances. Here are some examples of these substances: *Lactobacillus reuterii*, which is a member of normal microflora of human and many other animals, produce a low molecular weight antimicrobial substance reuterin; subspecies of *Lactococcus lactis* produce a class I bacteriocin, nisin A; *Enterococcus feacalis* DS16 produces a class I bacteriocin cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin plantaricin S; *Lactobacillus acidophilus* produces a class III bacteriocin acidophilicin A. (Quwehand and Vesterlund 2004) Production of bacteriocins is highly affected by the factors of the species of microorganisms, ingredients and pH of medium, incubation temperature and time. Nisin, produced by L. lactis subsp. lactis is the wellknown bacteriocin and it is allowed to use in food preparations. (Çakır 2003)

Lactobacilli and Bifidobacteria isolated from human ileum were assayed if they have antimicrobial activity against a range of indicator microorganisms, *Listeria, Bacillus, Enterococcus, Staphylococcus, Clostridium, Pseudomonas, E. coli, Lactobacillus, Streptococcus, Bifidobacterium* and *Lactococcus*. Antimicrobial activity of *Lactobacillus salivarius* UCC118 was counted against to these bacteria listed above. The study showed that
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Lactobacillus salivarus UCC118 is significantly capable of inhibiting in vitro growth of both some gram positive and some gram negative bacteria such as, L. fermentum KLD, B. longum, B. bifidum, Bacillus subtilus, B. Cereus, B.thuringiensis, E. faecalis, E. faecium etc. although it is not effective against some of Lactobacillus, Lactococcus, Leuconostoc, Streptococcus etc. species. (Dunne et al., 1999)

Some milk products were used to isolate potential probiotic bacteria and determination of their possible antimicrobial activities. Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Serratia marcescens and Candida albicans were used as indicator microorganisms. After the study, the results showed that, Yakult and Ski D’ Lite probiotics inhibited all of the test indicator microorganisms, Nestle yogurt probiotics were bactericidal for S.aureus and P.aeruginosa but inhibitory for S. typhi , Neslac probiotics killed E. coli and S. typhi while they were only inhibitory for S.aureus and C. albicans, Gain probiotics inhibited C. albicans. (Chuayana et al., 2003)

In another study eight lactic acid bacteria strains producing bacteriocins were isolated from Burkina Faso fermented milk and they were examined for the antimicrobial activity against Enterococcus faecalis 103907 CIP, Bacillus cereus 13569 LMG, Staphylococcus aureus ATCC 25293, Escherichia coli 105182 CIP. The lactic acid bacteria strains were identified as Lactobacillus fermentum, Pediococcus spp., Lactococcus spp., Leuconostoc mesenteroides subsp. mesenteroides. The diameters of inhibition zones were obtained between 8 mm and 12 mm. Lactobacillus fermentum (S1) gave the biggest zone around 12 mm on Enterococcus faecalis while the smallest one is obtained from Leuconostoc mesenteroides subsp. mesenteroides (S5) on the same strain Enterococcus faecalis. (Savadogo et al., 2004)

In a research which was aimed to test the production of bacteriocin in vaginal lactobacilli flora and characterization of this flora was also made. First antimicrobial activity was assayed for 100 vaginal lactobacilli isolates. Six of them were determined for the production of bacteriocin. In this study, common human pathogens Gardnerella vaginalis, Pseudomonos aeroginosa, Proteus vulgaris, Escherichia coli, Enterobacter cloacae, Streptococcus milleri, Staphylococcus aureus and Candida albicans were used as indicator microorganisms. Six of the strains had bacteriocin activity against eight of ten different Lactobacillus species an also S.milleri, P. vulgaris, P. aeroginosa, E. coli, E. cloacae and G. vaginalis. But none of isolated strains showed efficiency on test organisms S. aureus and C.
Yeasts of *Candida* and *Saccharomyces* have also been used as yeast strains in the food industry. Also some characteristics of bacteriocins were obtained from the research. (Karaoğlu et al., 2003)

In another research, potential probiotic lactobacilli strains (*L. reuteri, L. plantarum, L. mucosae, L. rossiae* strains) (from pig feces), used as additives in pelleted feeding, were examined according to their antibacterial activity against to *Salmonella typhimurium* ATCC 27164, *E. coli, C. perfringens* 22G, *S. aureus* ATCC 25923, *B. megaterium* F6, *L. innocua* DSM 20649 and *B. hyodysenteriae* ATCC 27164. Generally the cell free extracts of lactobacilli were able to inhibit all potential pathogens except *B. hyodysenteriae* ATCC 27164. The study showed that, neutralization and treatment with catalase affect the antibacterial activity a little. A similar study was conducted and in that study four *Lactobacillus* strains (*L. salivarus* CECT5713, *L. gasseri* CECT5714, *L. gasseri* CECT 5715 and *L. fermentum* CECT5716) isolated from human milk were investigated whether they have antimicrobial potential and for comparison *L. coryniformis* CECT5711 was used. All of the strains showed antibacterial properties against pathogenic bacteria (*Salmonella choleraesuis* CECT4155, CECT409 and CECT443, *Esherichia coli* CECT439 and *E. coli* O157:H7 serover CECT4076, *Staphylococcus aureus* CECT4013 and CECT9776, *Listeria monocytogenes* Scott A and the spoilage strain *Clostridium tyrobutyricum* CECT4011). However, the antimicrobial properties of lactobacilli strains varied and *L. Salivarus* CECT5713 revealed not only the best in vitro antibacterial activity, but also the highest protective effect against a *Salmonella* strain in the murine infection model. (Olivares et al., 2005)

Antimicrobial activity is thought to be an important means for Probiotics to competitively exclude or inhibit invading bacteria. (Carr et al., 2002; Roos and Holm 2002) Some do so by secreting non-specific antimicrobial substances, such as short-chain fatty acids (Carr et al., 2002) or hydrogen peroxide (Eschenbach et al., 1989), while others produce toxins with very narrow killing ranges, such as bacteriocins, bacteriocin-like inhibitory substances (BLIS), and bacteriophages. (Smith et al., 2007; Tagg and Dierksen 2003) Production of antimicrobial substances against pathogens has been proposed as one of the mechanisms by which they improve health. These substances include bacteriocins and organic acids, which inhibit a range of common and emerging food borne pathogens. (Opeyemi Daniel Amund, 2010)
Previous investigations have shown that certain strains of *Lactobacillus* species act as probiotic organisms within the gastrointestinal tract, helping to reduce colonization and infection with potential enteric pathogenic bacteria. Two potentially debilitating enteric pathogens, Entertoxigenic *Escherichia coli* and Enterohemorrhagic *Escherichia coli*, affect millions of people worldwide; however data on the effectiveness of probiotics for these strains of *E. coli* are minimal. In this study, we investigated the inhibitory effect of four *Lactobacillus* species against the growth of these two pathogenic strains of *E. coli*. The amount of growth inhibition was determined using both live lactobacilli cultures and supernatant obtained from actively growing lactobacilli broth cultures. The amount of growth inhibition of each *E. coli* strain varied depending on the method as well as the species of *Lactobacillus* used. Experimental findings suggest that Entertoxigenic *E. coli* was strongly inhibited when exposed to live lactobacilli cells, but only partially inhibited when exposed to supernatant alone. In contrast, Enterohemorrhagic *E. coli* was equally inhibited by exposure to live cells or supernatant. (Apella et al., 1990)

Urinary tract infection (UTI) is the most common bacterial infection seen in clinical practice. Human UTI comprises disease entities such as acute pyelonephritis with renal parenchymal involvement, cystitis limited to the urinary bladder, and asymptomauria. (W. E. McKevitt M., 1985) Enterobacteriaceae such as *Escherichia coli*, which are normal inhabitants of human intestines, account for the vast majority of these uncomplicated infections. (Genital areas are therefore recommended for prevention of UTI. On the other hand, studies have shown a correlation between a loss or disruption of the normal genital microflora, in particular *Lactobacillus* species, and an increased incidence of genital and bladder infections. (Redondo-Lopez et al., 1990) Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial micro flora. Preclinical and clinical reports have focused on lactobacillus strains, their possible prophylactic effects against experimental *E. coli* infection, and the use of these strains for the prevention of human urogenital infections.

Suitable animal experimental models are required for appropriate preclinical studies of UTIs. Hagberg et al. were the first to show that mice could be challenged intravesically (by introducing pathogens directly into the bladder) without further manipulations of the urinary tract (Hagberg L. et al., 1983) and the murine model of ascending pyelonephritis has served as an excellent tool for defining the roles of individual virulence factors in the pathogenesis of UTI. (Hopkins W. J. et al., 1998)
Time course and host responses to *Escherichia coli* urinary tract infection in genetically distinct mouse strains. It should be noted, however, that the inoculums doses used in murine models are very high (10^8 CFU). Furthermore, high bladder infection levels reportedly persisted over the 14-day study period only in C3H/HeJ and C3H/OuJ mice, which are lipopolysaccharide (LPS) nonresponder strains, while strains such as C3H/HeN, C57BL/6, BALB/c, DBA.1, DBA.2, and AKR showed progressive resolution of bladder infections over a 14-day period. *(Hopkins W. J. et al., 1998)*

Intraurethral treatment with *L. casei* Shirota (10^8 CFU/day) inhibited pathogen growth in the urinary tract and suppressed infection-induced inflammatory responses. The characteristics of this antimicrobial activity included (i) a heat-killed (HK) preparation of *L. casei* Shirota effectively lowering levels of infectious bacteria and (ii) effectiveness of treatment during the post infection period. These results suggest that the probiotic *L. casei* strain Shirota is potentially useful for both preventive and therapeutic treatment of UTI. This review considers whether probiotics are effective agents for the treatment and/or prevention of bacterial vaginosis (BV). There seems to be an association between the absence of, or low concentrations of, vaginal lactobacilli and the development of BV. Many studies have suggested that the presence of H2O2-producing vaginal lactobacilli may protect against BV, although some studies do not support this hypothesis. In-vitro studies have suggested that certain specific strains of lactobacilli are able to inhibit the adherence of Gardnerella vaginalis to the vaginal epithelium and/or produce H2O2, lactic acid and/or bacteriocins, which inhibit the growth of bacteria causing BV. Concerning the effectiveness of the administration of lactobacilli for the treatment of BV are mostly positive, it cannot yet be concluded definitively that probiotics are useful for this purpose. *(Falagas M.E. et al., 2007)*

A strain of *Lactobacillus*, identified as *Lactobacillus fermentum* L23, was selected from among 100 strains isolated from vaginal swabs of healthy, non-pregnant, pre-menopausal women. *L. fermentum* L23 was chosen on the basis of its bacteriocinogenic ability and its properties relevant to colonization, i.e. self-aggregation, adherence to vaginal epithelial cells and co-aggregation with bacterial pathogens. The antimicrobial preventative and curative effects produced by the probiotic *L. fermentum* L23 administered locally against *Escherichia coli* in a murine vaginal tract infection model were studied. Treatment with *L. fermentum* L23 during the post-infection period showed complete inhibition of pathogen growth from day 5. Thus, this in vivo study indicated that the probiotic bacterium *L.*
*fermentum* L23 produced both preventative and curative effects on *E. coli* growth. The beneficial properties and the production of antimicrobial metabolites may act in situ to inhibit a pathogenic micro-organism within the vaginal environment. Strain L23 could be a good natural alternative to other therapies used for genital infections. *(Pascual L. et al., 2010)*

Lactic acid bacteria (LAB) are generally regarded as safe and their antimicrobial peptides (bacteriocins) have been used in the preservation of many food products. Various claims have been attributed to LAB with probiotic properties, e.g. reduction or prevention of gastrointestinal disorders, including inflammatory bowel disease, alleviation of lactose intolerance, lowering of serum cholesterol levels, and stimulation of the immune system and control of tumour growth. Some probiotic claims may be associated with the production of antimicrobial peptides (bacteriocins).

The inhibitory mechanism was found to be dependent on the lowering of the pH of the medium and production of lactic acid. The antibacterial activity of *L. paracasei* 17 and *L. casei* 299 from NCDC were more effective in inhibiting *Salmonella enteritidis*, within 8hrs contact of fermented whey. Among various species, *Lactobacillus acidophilus* *(Gandhi and Nambudripad 1978), L. rhamnosus* *(Isolauri et al., 1995)*, *L. casei*, have been shown their efficacy in prophylactic management of acute diarrhoea in children, with an associated increase in the immunity.

Several studies have been carried out to investigate the types of antimicrobials produced by probiotics and the range of pathogens susceptible to them. Common antimicrobials produced include bacteriocins (antimicrobial proteinaceous substances, e.g. nisin), hydrogen peroxide and organic acids such as lactic and acetic acids. In vitro and *in vivo* studies by Wang et al. (2004) showed suppression of the pathogen *Helicobacter pylori* by *Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* BB12 contained in yoghurt. *(Opeyemi Daniel Amund 2010)*

Spent culture supernatant (SCS) of the probiotic *Lactobacillus rhamnosus* GG had been reported to exert antibacterial activity against *Salmonella typhimurium*. However, the chemical identity of the antimicrobial compound(s) responsible remained unknown. A survey of the antimicrobial compounds produced by *L. rhamnosus* GG was performed. *Lactobacillus rhamnosus* GG produced a low-molecular weight, heat-stable, non-proteinaceous bactericidal substance, active at acidic pH against a wide range of bacterial species. *(Sigrid C.J.et al., 2006)* Antimicrobial substances produced by lactic acid bacteria can be divided into two main
groups: low molecular mass substances with molecular mass <1000 Da and high molecular mass substances with molecular mass >1000 Da, such as bacteriocins. All non-bacteriocin antimicrobial substances from LAB are of low molecular mass. (J.W. Collins et al., 2010)

**BACTERIOCIN OR ANTIMICROBIAL PROTEIN PURIFICATION**

Bacteriocins were first identified almost 100 years ago as a heat-labile product present in cultures of *Escherichia coli V* and toxic to *E. coli S* and were given the name of colicin to identify the producing species. (Gratia 1925) Fredericq demonstrated that colicins were proteins and that they had a limited range of activity due to the presence or absence of specific receptors on the surface of sensitive cells. (Fredericq 1946) Since then, bacteriocins have been found in all major lineages of Bacteria and, more recently, have been described as universally produced by some members of the Archaea. (Riley and Wertz 2002a; Riley and Wertz 2002b; Shand and Leyva 2008) According to Klaenhammer, 99% of all bacteria may make at least one bacteriocin, and the only reason we have not isolated more is that few researchers have looked for them. (Klaenhammer 1999)

Two main features distinguish the majority of bacteriocins from classical antibiotics: bacteriocins are ribosomally synthesized and have a relatively narrow killing spectrum. The bacteriocin family includes a diversity of proteins in terms of size, microbial target, mode of action, release, and immunity mechanisms and can be divided into two main groups: those produced by Gram-negative and Gram-positive bacteria. (Gordon et al., 2006; Heng et al., 2007)

**Bacteriocins of Gram-negative bacteria**

Surveys of *E. coli*, *Salmonella enterica*, *Hafnia alvei*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* reveal levels of bacteriocin production ranging from 3 to 26% of environmental isolates. (Gordon et al., 2006; Riley et al., 2003) Colicins, bacteriocins produced by *E. coli*, are found in 30–50% of the strains isolated from human hosts and are often referred to as virulence factors. (Riley and Gordon 1992) Much higher levels of bacteriocin production have been found in some Gram-negative bacteria, such as *Pseudomonas aeruginosa*, in which >90% of both environmental and clinical isolates produce bacteriocins. (Michel-Briand and Baysse 2002)
Since their discovery, the colicins of *E. coli* have been the most extensively studied Gram-negative bacteriocins, and they now serve as a model system for investigating the mechanisms of bacteriocin structure/function, genetic organization, ecology, and evolution. *(Cascales et al., 2007)* Colicins are high molecular weight proteins that kill target cells through a variety of mechanisms. Nomura showed that colicins E1 and K inhibit macromolecular synthesis without arrest of respiration, colicin E2 causes DNA breakdown, and colicin E3 stops protein synthesis. *(Nomura 1967)* In each case, he showed that the lethal action is reversed by treatment with trypsin. Since his pioneering work, colicins were shown to kill their targets by either membrane permeabilization or nucleic acid degradation. *(Braun et al., 1994; Riley and Wertz 2002b; Smarda and Smajs 1998)*

Colicins are usually encoded on one of two types of colicinogenic plasmids. *(Pugsley and Oudega 1987)* Type A plasmids are small (6 to 10 kb) and present in numerous copies per cell. They are mobilizable in the presence of a conjugative plasmid and are amplifiable. Type B are monocopy plasmids of about 40 kb, which carry numerous genes in addition to those encoding colicin activity and are able to conjugate. However, plasmid carriage of bacteriocins is not a requirement. A close relative to the colicins, the bacteriocins of *Serratia marcescens*, are found on both plasmids and the chromosome. *(Ferrer et al., 1996; Guasch et al., 1995)* A colicin protein is comprised of three functionally distinct domains; receptor recognition, protein translocation, and killing. *(Cao and Klebba 2002)* In colicins, the central domain comprises about 50% of the protein and is involved in the recognition of specific cell surface receptors on the outer membrane of the target cell. *(Zakharov and Cramer 2004)*

In addition to colicins, *E. coli* strains produce a second type of bacteriocin, known as microcins, which are smaller than colicins and share more properties with the bacteriocins produced by Gram-positive bacteria, including thermostability, resistance to some proteases, relative hydrophobicity, and resistance to extreme pH. *(Baquero and Moreno 1984; Gillor et al., 2004; Pons et al., 2002)* Fourteen microcins have been reported to date, of which only seven have been isolated and fully characterized. However, these seven possess a diversity of killing mechanisms *(Duquesne et al., 2007a)*; some are active as unmodified peptides, while others are heavily modified by dedicated maturation enzymes. *(Duquesne et al., 2007b)*

The successful use of probiotics-producing colicins, microcins, or any other bacteriocins requires understanding the factors influencing the frequency of bacteriocin
production in a bacterial population. This aspect of bacteriocin ecology was recently studied in clinical and environmental *E. coli* populations. Recent evidence indicates that the frequency of bacteriocin production in *E. coli* populations can vary from 10 to 80% depending on the animal host from which they were isolated (Gordon et al., 1998; Gordon and O'Brien, 2006), the host's diet (Barnes et al., 2007), temporal changes (Gordon et al., 1998), and the type of bacteriocin produced by the strain. (Gordon et al., 2007) These observations suggest that it is not enough for antimicrobial-producing probionts to be proven potent against pathogens; they also need to complement the existing bacterial dynamics in the target host.

**Bacteriocins of Gram-positive bacteria**

Bacteriocins of Gram-positive bacteria are as abundant and even more diverse than those found in Gram-negative bacteria. The Gram-positive bacteriocins resemble many of the antimicrobial peptides produced by eukaryotes; they are generally cationic, amphiphilic, membrane-permeabilizing peptides, and range in size from 2 to 6 kDa. (Heng et al., 2007) They differ from bacteriocins of Gram-negative bacteria in two fundamental ways. (Riley and Wertz 2002b) First, the bacteriocins produced by Gram-positive bacteria are not necessarily lethal to the producing cell. This critical difference is due to dedicated transport mechanisms Gram-positive bacteria encode to release the bacteriocin toxin. Typically, their biosynthesis is self-regulated with specifically dedicated transport mechanisms facilitating release, although some employ the Sec-dependent export pathway. (Drider et al., 2006; Eijsink et al., 2002; Maqueda et al., 2008) Second, the Gram-positive bacteria have evolved bacteriocin-specific regulation, whereas bacteriocins of Gram-negative bacteria rely solely on host regulatory networks. (Nes et al., 1996)

Bacteriocins produced by LAB, which have a long history of use in fermentation and meat and milk preservation, are the best characterized of this group. (Cintas et al., 2001) Four classes of LAB antibiotics are identified: Class I is comprised of modified bacteriocins, known as lantibiotics (Twomey et al., 2002); class II includes heat stable, minimally modified bacteriocins (Drider et al., 2006; Eijsink et al., 2002); class III includes larger, heat-labile bacteriocins; and class IV is comprised of complex bacteriocins carrying lipid or carbohydrate moieties. (Heng et al., 2007) Classes I and II have been the focus of most probiotic research.
Lactic acid bacteria have been employed for centuries in the fermentation of food, partly due to the fact that they can prevent the growth of spoilage and pathogenic microorganisms. *(Cheigh and Pyun 2005)* They produce bacteriocins, the l antibiotics, so named because they are post-translationally modified to contain amino acids such as thioether bridges of l anthionine and 3-methyllanthionine or dehydroalanin. *(Twomey et al., 2002)* L antibiotics are ribosomally synthesized bacteriocins that target a broad range of Gram-positive bacteria and are subdivided into three groups on the basis of their structure and mode of action: Type A lantibiotics, such as nisin, are small (2–5 kDa), elongated, screw-shaped proteins that contain positively charged molecules, which kill via the formation of pores, leading to the dissipation of membrane potential and the efflux of small metabolites from the sensitive cells. *(Nagao et al., 2006)* Nisins have a dual mode of action: (1) They bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell death, and (2) they employ lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. *(Wiedemann et al., 2001)* Type B lantibiotics, such as mersacidin *(Twomey et al., 2002)*, kill by interfering with cellular enzymatic reactions, such as cell wall synthesis. *(Pag and Sahl 2002; Sahl and Bierbaum 1998; Sahl et al., 1995)* Another subgroup is composed of two-component lantibiotics, such as lacticin 3147 *(Wiedemann et al., 2006)* consisting of two lantibiotic peptides that synergistically display antimicrobial activity. *(Ryan et al., 1998)* It was shown that the dual activities could be distributed across two peptides: While one resembles type B lantibiotic mersacidin, which depolarizes the membrane, the other is more similar to the type A lantibiotic class pore formers. *(Martin et al., 2004)*

Class II LAB bacteriocins are also small nonlanthionine-containing peptides. *(Drider et al., 2006; Oppegård et al., 2007)* The majority of bacteriocins in this group kill by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria. These bacteriocins are organized into subgroups: Class IIa is the largest group and its members are distinguished by shared activity against *Listeria* and a conserved amino-terminal sequence (YGNGVXaaC) that is thought to facilitate nonspecific binding to the target surface. Like type A lantibiotics, class IIa bacteriocins act through the formation of pores in the cytoplasmic membrane. Examples include pediocin (this group is also called pediocin-like bacteriocins), sakacin A, and leucocin A. *(Drider et al., 2006; Hechard and...*
Class IIb bacteriocins such as lacticin F and lactococcin G form pores, composed of two different proteins, in the membrane of their target cells. 

A third subgroup (IIc) has been proposed, which consists of bacteriocins that are sec-dependent, such as acidocin 1B. 

Class III bacteriocins are large heat-labile proteins such as helveticins J or lactacin B. 

An additional proposed class (IV) requires lipid or carbohydrate moieties for activity. Little is known about the structure and function of this class. Examples include leuconocin S and lactocin 27. 

Gram-positive bacteriocins, in general, and lantibiotics, in particular, require many more genes for their production than do those of Gram-negative bacteria. 

The nisin gene cluster, for example, includes genes for the prepeptide (nisA), enzymes for modifying amino acids (nisB, nisC), cleavage of the leader peptide (nisP), secretion (nisT), immunity (nisI, nisFEG), and regulation of expression (nisR, nisK). These gene clusters are most often encoded on plasmids but are occasionally found on the chromosome. 

Several Gram-positive bacteriocins, including nisin, are located on transposons. 

The conventional wisdom about the killing range of Gram-positive bacteriocins is that they are restricted to killing other Gram-positives. The range of killing can vary significantly, from relatively narrow as in the case of lactococcins A, B, and M, which have been found to kill only Lactococcus, to extraordinarily broad. 

For instance, some type A lantibiotics, such as nisin A and mutacin B-Ny266, have been shown to kill a wide range of organisms including Actinomyces, Bacillus, Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactococcus, Listeria, Micrococcus, Mycobacterium, Propionibacterium, Streptococcus, and Staphylococcus. 

Contrary to conventional wisdom, these particular bacteriocins are also active against a number of medically important Gram-negative bacteria including Campylobacter, Haemophilus, Helicobacter, and Neisseria. 

Production of bacteriocins in Gram-positive bacteria is generally associated with the shift from log phase to stationary phase. For example, nisin production begins during mid-log phase and increases to a maximum as the cells enter stationary phase.
Kruijff 1999) The regulation of expression is not cell cycle dependent, per se, but rather culture density dependent. (Dufour et al., 2007) It has been demonstrated that nisin A acts as a protein pheromone in regulating its own expression, which is controlled by a two-component signal transduction system typical of many quorum-sensing systems. (Hechard and Sahl 2002) The genes involved are nisR (the response regulator) and nisK (the sensor kinase). Nisin transcription is induced by the addition of nisin to the culture medium, with the level of induction directly related to the level of nisin added. (Kuipers et al., 1995)

The human GI tract is a complex ecosystem in which a delicate balance exists between the intestinal microflora and the host. The microflora serves as a primary stimulus for the development of the mucosal immune system. (Deplancke and Gaskins 2002; Macfarlane and Cummings 2002) Two main genera of lactic acid bacteria dominate the intestinal flora, including 56 species of Lactobacillus and numerous species of Bifidobacterium. Most of these species have been shown to produce bacteriocins in vitro. (Avonts and De Vuyst 2001; Carr et al., 2002; Cross 2002) More recently, some of these strains have also been shown to produce bacteriocins in vivo. One particularly compelling study demonstrated the in vivo activity of Lactobacillus salivarius strain UCC118, which produces a potent broad-spectrum bacteriocin (Abp118) active against the food-borne pathogen Listeria monocytogenes. (Claesson et al., 2006) In mice, the L. salivarius strain provided protection against L. monocytogenes infection, while a mutant strain of the same species, impaired in its bacteriocin production ability, did not. Even more compelling, the bacteriocin-producing strain provided no protection against pathogen infection when mice were infected with a strain of L. monocytogenes expressing the cognate Abp118 immunity protein. (Corr et al., 2007)

A strain of Lactobacillus casei L26 LAFTI was shown to significantly inhibit an enterohemorrhagic strain of E. coli and a strain of L. monocytogenes in mice (Su et al., 2007a, b), probably due to bacteriocin production (Pidcock et al. 2002). The release of bacteriocins inhibiting Helicobacter pylori, a human pathogen that causes severe gastroduodenal diseases (Kandulski et al., 2008), has been chiefly studied in lactobacilli strains. A BLIS with anti-H. pylori activity was identified in probiotic Lactobacillus johnsonii strain LA1 (Gotteland et al., 2008; Michetti et al., 1999) and Lactobacillus acidophilus strain LB. (Coconnier et al., 1998) In both cases, the inhibitory activity was retained when H. pylori was bound to intestinal epithelial cells. Oral administration of L.
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*acidophilus* LB in mice protected the animals from infection with *Helicobacter felis.* (Coconnier et al., 1998; Nedrud and Blanchard 2001) This PB was further shown to inhibit gastric colonization and prevent the development of gastric inflammation. (Coconnier et al., 1998) Administration of *L. johnsonii* LA1 supernatant to adult patients colonized by *H. pylori* significantly decreased infection (Gotteland et al., 2008; Gotteland and Cruchet 2003), while oral consumption of the live bacteria by school children, which were found to be *H. pylori* positive, resulted in a significant decrease in urease production. (Cruchet et al., 2003) Mutacin B-Ny266, a lantibiotic produced by *Streptococcus mutans,* was recently shown to inhibit a broad spectrum of multi-resistant pathogens including staphylococci, streptococci, and *Neisseria* strains (Mota-Meira et al., 1997, 2000; Parrot et al., 1990) and was found active against methicillin-resistant *Staphylococcus aureus* when assayed in a mouse model. (Mota-Meira et al., 2005)

Most of the members of class IIa bacteriocins have relatively narrow killing spectra compared to those in class I and inhibit only closely related Gram-positive bacteria. (Heng et al., 2007) However, there are exceptions, such as pediocin, which has a fairly broad inhibitory spectrum and can inhibit *Streptococcus aureus* and vegetative cells of *Clostridium* spp. and *Bacillus* spp. and *Listeria.* (Cintas et al., 1997; Eijsink et al., 2002; Nes and Holo, 2000; van Reenen et al., 1998) A pediocin-producing strain of *Pediococcus acidilactici,* able to survive in the GI tract, was recently isolated and found to be an effective inhibitor of several Gram-positive bacterial pathogens, such as *Enterococcus* spp. (including vancomycin-resistant strains) and *L. monocytogenes.* Furthermore, it inhibited gastric adhesion of opportunistic pathogens from *Klebsiella,* *Pseudomonas,* and Shigella genera. (Piva and Casadei 2006; Speelmans et al., 2006) Another promising probiont is the bacteriocin producer *Enterococcus mundtii* strain ST4SA, active against a number of Gram-positive bacteria, including *Enterococcus faecalis,* *Streptococcus pneumoniae,* and *Staphylococcus aureus,* as well as the Gram-negative bacteria *P. aeruginosa* and *K. Pneumonia.* (Granger et al., 2008) The survival, persistence, and bacteriocin production of this strain were successfully evaluated within the GI tract of pigs.

Weakness of the bacteriocins produced by Gram-positive bacteria, with respect to their use in probiotic applications, is that they seldom inhibit commonly encountered enteropathogenic bacteria such as *Enterobacter,* *Klebsiella,* or *Salmonella.* However, bacteriocins produced by Gram-negative bacteria can accomplish this task. For example, *E.*
coli strain H22 inhibited the growth of seven genera of the family Enterobacteriaceae (Enterobacter, Escherichia, Klebsiella, Morganella, Salmonella, Shigella, and Yersinia). The observed inhibition was attributed to the production of microcin C7 (Smajs et al., 2008) and colicins E1 and Ib, as well as aerobin and an unidentified phage. (Cursino et al., 2006) Simultaneous administration of the probiont and the enteric pathogen Shigella flexneri to germ-free mice resulted in a strong inhibition of the pathogen, which was attributed to its microcin production. (Cursino et al., 2006) A more widely used enteric probiont is E. coli strain Nissle 1917, originally isolated from the feces of a soldier who did not develop diarrhea during a severe outbreak of shigellosis. (Snelling 2005) Some of the beneficial properties of this strain may be attributable to bacteriocin production, as this strain was shown to produce two microcins, H47 and M. (Patzer et al., 2003)

The bacteriocin activity includes other species of lactobacilli, as well as a variety of Gram-positive and Gram-negative aerobic, facultative, and obligate anaerobic bacteria. It is significant that one species of Lactobacillus will produce a bacteriocin that inhibits the growth of other lactobacilli. This may be one mechanism that allows Lactobacillus to dominate the ecosystem by suppressing not only other bacteria but also other lactobacilli. This in turn reduces competition within an ecosystem.

Although bacteriocins may be found in many Gram-positive and Gram-negative bacteria, those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives. Bacteriocins produced by LAB are small, ribosomally synthesized, antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocin(s). (De Vuyst 2007)

An adhesion-promoting protein involved in the binding of Lactobacillus fermentum strain 104R to small intestinal mucus from piglets and to partially purified gastric mucin was isolated and characterized. Spent culture supernatant fluid and bacterial cell wall extracts were fractionated by b and gel filtration. The active fraction was purified by affinity chromatography. The adhesion-promoting protein was detected in the fractions by adhesion inhibition and dot blot assays and visualized by polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate-PAGE. (Maurilia R. et al., 2002)

Lactobacillus acidophilus 30SC was tested for its potential as a probiotic culture. The strain produced a heat-stable antimicrobial compound that was shown to be proteinaceous in
nature and, therefore, referred to as a bacteriocin. The bacteriocin was active over a wide pH range and inhibited a number of Gram-positive bacteria including Listeria ivanovii and pathogenic strains. The bacteriocin was purified by 50% ammonium sulfate precipitation followed by hydrophobic interaction column chromatography. The SDS-PAGE of the active fractions resulted in a single band with estimated molecular mass of 3.5 kDa.

Bacteriocin producing Lactobacillus acidophilus strain was isolated from the gut of marine prawn (Penaeus monodon). This bacteriocin has broad range of antibacterial activity against major food born pathogens. Maximum bacteriocin production was observed at temperature 50°C, pH 4 and 0.9% sodium chloride & it was purified by ammonium sulphate precipitate. Dialysis was followed in a tubular cellulose membrane (1000 cut off) against 2L distilled water for 24 h, Molecular size of bacteriocin was determined using b gel following the procedure of Sambrro et al. (2006) was 2.5 KDa. This study revealed the possibility of using bacteriocin as a food preservative and the L. acidophilus strain as probiotic.

Bacteriocin producing Lactobacillus lactis strain isolated from marine environment, showed broad range of antibacterial activity against some major food borne pathogens. Maximum bacteriocin production was observed at 30°C, pH 6.0 and 1.5% sodium chloride solution. The crude bacteriocin was precipitated with 80% ammonium sulphate. The precipitate was dialysed against 20 mM potassium phosphate buffer (pH 7.0) for 12 h at 4°C. The molecular weight of the bacteriocin was determined by 15% SDS-PAGE, was 94 kDa. The study revealed the possibility of using bacteriocin as a food preservative and the L. lactis strain as probiotic. (G. Rajaram. et al., 2010)

MOLECULAR IDENTIFICATION OF PROBIOTIC STRAINS

Methods used for detection of probiotics in human gastrointestinal tract are identification of colony morphology, fermentation patterns, serotyping or some combination of these. Although these traditional methods have limitations they are used for identification. With the developing technology about the molecular typing it is getting more reliable to identify and differentiate bacterial strains. Classical microbiological techniques are really important for selection, enumeration and biochemical characterization (fermentation profiles, salt-pH-temperature tolerances) but it is not efficient to classify a culture taxonomically. Molecular characterization methods are powerful even between closely related species. There are number of alternative taxonomic classification methods well known including
hybridization with species-specific probes and generation of profile PCR applicants by species-specific primers. *(Klaenhammer and Kullen 1999)* Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and RAPD) are mainly used as molecular tools. *(Bulut 2003)* Comparison between these methods, the most powerful and accurate one is sequencing. *(Coeuret et al., 2003)*

Characterization of microorganisms according to their 16S rDNA regions sequencing was firstly proposed by Woese in 1987. The application of 16S or 23S rRNA-targeted oligonucleotide probes is the best and most reliable approach to identify bacteria on a phylogenetic basis. The 16S rRNA gene is nearly 1540 bases long and includes variable regions while the general structure is highly conserved. Because the probes have the broadest specificity ranging from universal to species specificity, it is possible to use 16S rRNA gene to study phylogenetic relationships between microorganisms and identify them more accurately. *(Çakır 2003, Holzapfel et al., 1998, Charteris et al., 1997)*

In one study, the PCR-ARDRA technique was used to identify potential probiotic *Lactobacillus* species isolated from bovine vagina. 16S rRNA gene was amplified by PCR and products were digested with four restriction enzymes (*Sau* 3AI, *Hinf* I, *Hinc* II and *Dra* I). Most of the digestion profiles obtained from the amplified 16S rDNA gene of these strains agreed with the theoretical profile matching with *Lactobacillus fermentum*. Among all strains, four homofermentative lactobacilli showed a restriction profile that matched with *Lactobacillus gasseri* and a facultative heterofermentative strain was identified as *Lactobacillus rhamnosus*. *(Otero et al., 2006)*

Restriction enzyme analysis were done by using pulsed with gel electrophoresis (REA-PFGE) and intergenic transcribed spacers (ITS)-PCR restriction fragment length polymorphism (RFLP) techniques for identification of probiotic potential strains (by sequencing of the 16S rRNA gene) isolated from koko and koko sour water (African spontaneously fermented millet porridge and drink). *Taq* I and *Hae* III restriction enzymes were used for digestion. From the result of ITS-PCR RFLP, four groups were obtained including group 1 *Weisella confuse*, group 2 *Lactobacillus fermentum*, group 3 *Lactobacillus salivarus* and group 4 *Pediococcus* spp. At the end it was showed using for identification of these strains the ITS-PCR RFLP technique, 16S rRNA gene sequencing is very reliable. *(Lei and Jakobsen 2004)*
To identify lactobacilli used as starter and probiotic cultures, amplified ribosomal DNA restriction analysis (ARDRA) was applied. Firstly group-specific and species-specific 16S rDNA primers were used to amplification. Cfo I, Hinf I, Tru 91 and ScrFI restriction enzymes were selected for digestion. The results revealed three groups: A, B and C. It is suggested that ARDRA by using Cfo I was reliable method for differentiation of *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *Lactis*. *(Roy et al., 2001)*

Some researchers aimed to develop a novel multiplex PCR primer set to identify seven probiotic Lactobacillus species (*L. acidophilus, L. delbrueckii, L. casei, L. gasseri, L. plantarum, L. reuteri* and *L. rhamnosus*). The primer set containing seven specific and two conserved primers, was obtained from the integrated sequences of 16S and 23S rRNA genes and their rRNA intergenic spacer region of each species. 93.6% accuracy was obtained to identify the seven target species. The study showed that the multiplex primer set is really efficient tool for simple, rapid and reliable identification of *Lactobacillus* species. *(Kwon et al., 2004)*

In another study potential probiotic *Lactobacillus* strains isolated from human, animal and food were identified by 16S-23S rRNA restriction profiling at species level. Firstly PCR amplification of 16S-23S rRNA intergenic spacers was done by using universal primers. It is followed by digestion of PCR products by 11 restriction enzymes with 6bp specificities. Some of the enzymes were *Sfi* I, *Hind* III, *Dra* I, *EcoRI, EcoRV* etc. the study was concluded that identification could be done by DNA fingerprints generated by restriction endonucleases. The amplified ribosomal DNA restriction analysis (ARDRA) was an easier, faster and more accurate method. *(Moreire et al., 2005)*