CHAPTER 2

LITERATURE REVIEW

2.1 Definition of probiotics

The probiotic concept has been defined by Fuller (1989) to mean “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. Salminen et al. (1999) proposed that probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host.

Probiotics have been also defined as: “mono- or mixed culture of live microorganisms that applied to animal or man, affect beneficially by the host by improving the properties of the indigenous microflora” (Havenaar et al., 1992). Probiotics are nonpathogenic and non-toxigenic, retain viability during storage, and survive passage through the stomach and small bowel (MacFarland and Cumming, 1999).

2.2 The benefit roles of probiotics

Several beneficial functions have been reported for probiotic bacteria, e.g. vitamin production, production of important digestive enzymes, prevention and treatment of diarrhea, establishment of a healthy flora in premature babies, alleviation of the symptoms of lactose maldigestion, stimulation of the immune system, suppression of tumorigenesis, and cholesterol reduction (Holzapfel and Schillinger, 2002; O’Sullivan, 2001).

It is well known that the presence of probiotics is important for the maintenance of the intestinal microbial ecosystem. Probiotics have been shown to possess inhibitory activity toward the growth of pathogenic bacteria, including *Bacillus cereus, Campylobacter jejuni, Clostridium difficile, Clostridium perfringens, Escherichia coli, Listeria monocytogenes, Listeria innocua, Neisseria gonorrhoeae, Salmonella typhimurium, Salmonella enteritidis, Staphylococcus aureus,* and *Vibrio cholerae* (Audisio et al., 1999; Audisio et al., 2000; Bogovic-Matijastic et al., 1998; Lee et al., 2003; Mante et al., 2003; Ocaña et al., 1999a; Ocaña et al., 1999b;Quan and Harsharnjit, 2001; Suma et al., 1998, Waard et al., 2002). This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocin, or reuterin or to competitive adhesion to the epithelium. Moreover, probiotic strains can produce β-galactosidase which improve tolerance to lactose. It is useful for the symptoms of lactose maldigestion (Fooks et al., 1999; Holzapfel and Schillinger, 2002; O'Sullivan, 2001)

Probiotic studies have suggested that the probiotic consumption associated with anticarcinogenic, antimutagenic, and antitumorigenic activities. However no definitively successful clinic trials using probiotics in cancer therapy have been carried out (Fooks et al., 1999; Holzapfel and Schillinger, 2002; O'Sullivan, 2001)

2.3 Microorganisms used as probiotics

Currently available probiotic preparations contain *Lactobacillus delbreuckii* subsp.bulgaricus, *L. acidophilus, L. casei, L. fermentum, L. plantarum, L. brevis, L. cellobiosus, L .lactis* and *L. reuteri.* The bifidobacteria currently used as probiotics are *Bifidobacterium* adolescentis, *B. animalis, B. bifidum, B. infantis, B. longum* and *B.*

thermophilum. The first use of streptococci as probiotics was in the form of soured milk and yoghurt. The yoghurt starter *Streptococcus salivarius* subsp.*thermophilus* is still common probiotic organism. Probiotics also contain bacteria belonging to the genera *Leuconostoc, Pediococcus, Propionibacterium* and *Bacillus*. Yeasts (*Saccharomyces cerevisiae* and *Candida pintolopesii*) and moulds (*Aspergillus niger* and *A.oryzae*) are also used but mainly in animal products (Fuller, 1992). Examples of lactic acid bacteria used as probiotics for human consumption are *Lactobacillus delbreuckii* subsp.bulgaricus, *L. acidophilus, L. casei, L. rhamnosus, L. reuteri. Bifidobacterium bifidum, B. infantis, B. longum* and *B. breve, Streptococcus salivarius* subsp.*thermophilus, Enterococcus faecalis* and *E. Faecium* (Fooks et al., 1999).

2.4 Lactic acid bacteria (LAB) as probiotics

2.4.1 Lactic acid bacteria

Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB (Ercolini et al., 2001; Holzapfel et al., 2001; Jay, 2000; Stiles and Holzapfel, 1997). Lactic acid-producing Gram-positive bacteria but belonging to the phylum Actinobacteria are genera such as *Aerococcus, Microbacterium,* and *Propionibacterium* (Sneath and Holt, 2001) as well as *Bifidobacterium* (Gibson and Fuller, 2000; Holzapfel et al., 2001). Members of LAB share the property of being Gram-positive bacteria (Fooks et al., 1999) that ferment carbohydrates into energy and lactic acid (Jay, 2000). Depending on the organism, metabolic pathways differ

when glucose is the main carbon source: homofermentative bacteria such as *Lactococcus* and *Streptococcus* yield two lactates from one glucose molecule, whereas the heterofermentative (ie. *Leuconostoc* and *Weissella*) transform a glucose molecule into lactate, ethanol and carbon dioxide (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers et al., 2000). In addition, LAB produce small organic compounds that give the aroma and flavor to the fermented product (Caplice and Fitzgerald, 1999).

The taxonomy of LAB based on comperative 16S ribosomal RNA (rRNA) sequencing analysis has revealed that some taxa generated on the basis on phenotypic features do not correspond with the phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR) based methods, such as rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) as well as pulse-field gel electrophoresis (PFGE), are regarded important for specific characterization and detection of LAB strains (Gevers et al., 2001; Holzapfel et al., 2001). Recently, culture-independent approaches have been applied for the detection of intestinal microbiota (Zoetendal et al., 2002). Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) analysis of faecal 16S rDNA gene and its rRNA amplicons have shown to be powerful approaches in determining and monitoring the bacterial community in faeces (Zoetendal et al., 1998).

LAB were first isolated from milk (Carr et al., 2002; Sandine et al., 1972) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Harris et al., 1992; Gobbetti and Corsetti, 1997; Jay, 2000; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan et al., 2002). LAB occur naturally in fermented food (Caplice and Fitzgerald, 1999) and have been detected in soil, water,

manure and sewage (Holzapfel et al., 2001). LAB exist in human (Boris et al., 1998; Carroll et al., 1979; Eideman and Szilagyi, 1979; Elliott et al., 1991; Martín et al., 2003; Ocaña et al., 1999; Reid, 2001; Schrezenmeir and de Vrese, 2001) and in animal (Fujisawa and Mitsuoka, 1996; Fuller and Brooker, 1974; Gilliland et al., 1975; Klijn et al., 1995; Sandine et al., 1972; Schrezenmeir and de Vrese, 2001). However, some LAB are part of the oral flora which can cause dental caries (Monchois et al., 1999; Sbordone and Bortolaia, 2003). LAB can work as spoilage organisms in foods such as meat, fish and beverages (Jay, 2000; Liu, 2003). LAB have been used as a flavoring and texturizing agent as well as a preservative in food for centuries and are now added as starters in food (Caplice and Fitzgerald, 1999). LAB, such as lactobacilli, *L. lactis*, and *Streptococcus thermophilus*, inhibit food spoilage and pathogenic bacteria and preserve the nutritive qualities of raw food material for an extended shelf life (Heller, 2001; O'Sullivan et al., 2002). Recently, the use of metabolites of LAB as biological preservatives in food packaging materials has been discussed (Pirttijärvi et al., 2001; Scannell et al., 2000).

LAB play an important role in processing animal feeds like silage (Aukrust and Blom, 1992; Driehuis and Oude Elferink, 2000; Holzer et al., 2003). The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, causing the pH of the growth environment to decrease (Caplice and Fitzgerald, 1999; Kuipers et al., 2000). Low pH induces organic acids to become lipid soluble and diffuse through the cell membrane into the cytoplasm (Gottschalk, 1988). LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Caplice and Fitzgerald, 1999; De Vuyst and Degeest, 1999; Rodrígues et al., 2003), some of which may act as antimicrobials.

2.4.2 Antimicrobial compounds produced by lactic acid bacteria

2.4.2.1 Organic acids

Fermentation by LAB is characterized by the accumulation of organic acids and the accompanying reduction in pH. The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lindgren and Dobrogosz 1990). The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules (Gould 1991, Podolak et al. 1996). It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane (Kashket 1987). The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Smulders et al., 1986; Earnshaw 1992).

Lactic acid is the major metabolite of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts. However, different microorganisms vary considerably in their sensitivity to lactic acid. At pH 5.0 lactic acid was inhibitory toward spore-forming bacteria but was ineffective against yeasts and moulds (Woolford, 1975). It was possible to grow *Aspergillus parasiticus* NRRL 2999 in a medium containing 0.5 or 0.75% lactic acid at pH 3.5 or 4.5 (El-Gazzar et al., 1987). Lindgren and Dobrogosz (1990) showed that at different pH ranges the minimum inhibitory concentration (MIC) of the undissociated lactic acid was different against *Clostridium tyrobutyricum*, *Enterobacter* sp. and

Propionibacterium freudenreichii ssp. *shermanii*. In addition, the stereoisomers of lactic acid also differ in antimicrobial activity, L-lactic acid being more inhibitory than the D-isomer (Benthin and Villadsen, 1995).

Acetic and propionic acids produced by LAB strains through heterofermentative pathways, may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang et al., 1986). They are more antimicrobially effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percent of undissociated acids than lactic acid at a given pH (Earnshaw, 1992). Acetic acid was more inhibitory than lactic and citric acids toward *Listeria monocytogenes* (Ahmad and Marth, 1989, Richards et al., 1995), and toward the growth and germination of *Bacillus cereus* (Wong and Chen, 1988). Acetic acid also acted synergistically with lactic acid; lactic acid decreases the pH of the medium, thereby increasing the toxicity of acetic acid (Adams and Hall, 1988).

2.4.2.2. Hydrogen peroxide

Hydrogen peroxide **(**H_2O_2) is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine hydroxy dinucleotide (NADH) peroxidase. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability (Kong and Davison, 1980). H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide (O_2^-) and hydroxyl ($OH^\cdot$) radicals which can damage DNA (Byczkowski and Gessner, 1988). It has been reported that the

production of H_2O_2 by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus*, *Pseudomonas* sp. and various psychotrophic microorganisms in foods (Davidson et al. 1983, Cords and Dychdala 1993). In raw milk, H_2O_2 activates the lactoperoxidase system, producing hypothiocyanate ($OSCN^-$), higher oxyacids (O_2SCN^- and O_3SCN^-) and intermediate oxidation products that are inhibitory to a wide spectrum of Gram-positive and Gram-negative bacteria (Reiter and Härnulv 1984; Conner, 1993).

2.4.2.3 Carbon dioxide

Carbon dioxide **(**CO_2) is mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability (Eklund, 1984). CO_2 can effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria (Farber, 1991; Hotchkiss et al., 1999). The degree of inhibition by CO_2 varies considerably between the organisms. CO_2 at 10% could lower the total bacterial counts by 50% (Wagner and Moberg, 1989), and at 20-50% it had a strong antifungal activity (Lindgren and Dobrogosz, 1990).

2.4.2.4 Aroma components

Diacetyl is produced by strains within all genera of LAB by citrate fermentation. The antimicrobial effect of diacetyl has been known since the 1930s (Jay, 1982). It inhibits the growth of Gram-negative bacteria by reacting with the

arginine-binding protein, thus affecting the arginine utilization (Jay, 1986). Jay (1982) showed that Gram-negative bacteria were more sensitive to diacetyl than Grampositive bacteria; the former was inhibited by diacetyl at 200 µg/mL and the latter at 300 µg/mL. Diacetyl at 344 µg /mL inhibited strains of *Listeria, Salmonella, Yersinia, Escherichia coli,* and *Aeromonas*. Since the production of diacetyl during lactic fermentation is low, e.g. 4 µg/mL produced by *Lactococcus lactis* ssp. *diacetylactis* (Cogan, 1980), and the acceptable sensory levels of diacetyl are at 2-7 µg /mL (Earnshaw, 1992), its practical use as a food preservative is limited. However, diacetyl may act synergistically with other antimicrobial factors (Jay, 1992) and contribute to combined preservation systems in fermented foods.

Acetaldehyde is produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* by the action of a threonine aldolase, which cleaves threonine into acetaldehyde and glycine. Since *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in yoghurt cannot metabolize acetaldehyde, it accumulates in the product at a concentration of about 25 ppm. Acetaldehyde at 10-100 ppm inhibits the growth of *Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* in dairy products (Piard and Desmazeaud, 1991).

2.4.2.5 Fatty acids

Under certain conditions, some lactobacilli and lactococci possessing lipolytic activities may produce significant amounts of fatty acids, e.g. in dry fermented sausage (Sanz et al., 1988) and fermented milk (Rao and Reddy, 1984). The antimicrobial activity of fatty acids has been recognized for many years. The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal

activity of fatty acids is dependent on chain length, concentration, and pH of the medium (Gould, 1991). The antimicrobial action of fatty acids has been thought to be due to the undissociated molecule, not the anion, since pH had profound effects on their activity, with a more rapid killing effect at lower pH (Kabara, 1993).

2.4.2.6 Bacteriocins

Some LAB strains ribosomally synthesize antimicrobial peptides, or bacteriocins, targeted to inhibit other Gram-positive bacteria (Abee, 1995; Barefoot and Nettles, 1993; Caplice and Fitzgerald, 1999; O'Sullivan et al., 2002). Even though antimicrobial peptides occupy an inhibition spectrum narrower than that of antibiotics (McAuliffe et al., 2001; Morency et al., 2001), bacteriocins produced by LAB have been reported to permeate the outer membrane of Gram-negative bacteria and to induce the inactivation of Gram-negative bacteria in conjunction with other enhancing antimicrobial environmental factors, such as low temperature, organic acid and detergents (Alakomi et al., 2000; Elliason and Tatini, 1999).

Bacteriocins produced by LAB are classified into three main groups, lantibiotics being the most documented and industrially exploited. The groups are lantibiotics (Class I), nonlantibiotics, small heat-stable peptides (Class II) and large heat-labile protein (Class III) (O'Sullivan et al., 2002).

Bacteria have self-protective mechanisms limiting the bacteriocin production, as in the case of nisin-producing *Lactococcus lactis* (Immonen and Saris, 1998; Kuipers et al., 1993; Qiao et al., 1995). The bacteriocin production is highest at the end of the exponential and early stationary phase (Daba et al., 1993; Thomas et al., 2000) and reduction is caused by proteolytic degradation of the bacteriocin (De Vuyst

and Vandamme, 1994; Thomas et al., 2000). Some bacterial strains, such as *Clostridium botulinum* 169B (Mazzotta and Montville, 1999) and *Streptococcus bovis* JB1 (Mantovani and Russell, 2001) are resistant to nisin. Resistance is assumed to be based on the enzymatic decomposition of nisin (Breuer and Radler, 1996). Nisin resistance in sporeforming strains has been associated with an enzyme produced during germination acting on the C-terminal lanthionine ring of nisin (Jarvis, 1967; Mazzotta and Montville, 1999).

Breuer and Radler (1996) demonstrated that differences in the resistance to nisin among *Lactobacillus casei* strains are related to cell-wall linked heteropolysaccharaides, whereas Mantovani and Russell (2001) reported nisin-resistant *Streptococcus bovis* JB1 cells having more lipoteichoic acid than nisin-sensitive cells.

In the last decade there has been an increasing in using LAB as probiotics. The important criteria for a LAB strain to be probiotic are, in addition to acid and bile tolerance, the ability to produce antimicrobial compound against pathogenic bacteria, and to adhere to and colonized human intestinal mucosa (Saxelin, 1995). LAB have a long history in biotechnology, especially in the manufacture and storage of food ingredients by fermentation processes. Some of the probiotic effects have been documented in clinical tests, *e.g.* the successful treatment of rotavirus diarrhea in small children by LAB administration (Isolauri et al., 1991). The mechanisms by which LAB exert beneficial health effects are not well understood. The ability to adhere to and colonize the intestinal or the urogenital tracts, even if transiently, are probably important factors that contribute to the survival of LAB and thus help them to induce positive health effects.

2.5 Prebiotics and their effects

A prebiotic has been defined as “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health”. Whereas, synbiotics have been defined as combination of probiotic and prebiotic (Gibson and Roberfroid, 1995). This combination might improve the survival of the bacteria crossing the upper part of the gastrointestinal tract, thereby enhancing their effects in large bowel. In addition, their effect might be additive or even synergistic (Roberfroid, 2000).

There are many prebiotic oligosaccharides known such as fructo-oligosaccharides, inulin, galacto-oligosaccharides, lactulose, lactosucrose, isomalto-oligosaccharides, soybean oligosaccharides, xylo-oligosaccharides, and gentio-oligosaccharides (Rastall and Maitin, 2002).

Several studies have attempted to use prebiotic for improving probiotics. Bielecka et al. (2002) studied the influence of fructan-type oligosaccharides (as prebiotics) on growth and acidify activity of *Bifidobacterium* strains (as probiotics) in vitro. The results showed that the majority of *Bifidobacterium* species utilized fructo-oligosaccharides and low-polymerized inulins, but only 18 out of 30 strains tested (mostly of *B. longum* and *B.animalis* species) were stimulated. Therefore, the researchers selected the stimulated strain of *B. longum* and *B. animalis*, oligofructose enhancing their growth as well as their synergistic pairs for in vivo studies on Wistar rats. *B. longum* slightly increased the fecal bifidobacteria live cell number, whereas *B. animalis* was not effective. But administration of bifidobacteria together with the prebiotic (as synbiotics) improved the bifidogenic effect.

Palframan et al. (2002) analyzed the effect of pH and dose on the growth of gut bacteria on commercial prebiotics in vitro. The results were found that both pH and dose altered the bacterial composition. It was observed that fructo-oligosaccharide and inulin demonstrated the greatest bifidogenic effect at pH 6.8 and 1% (w/v) carbohydrate, whereas galacto-oligosachharide, isomalto-oligosaccharide and lactulose demonstrated their greatest bifidogenic effect at pH 6 and 2% (w/v) carbohydrate.

The ability of a probiotic LAB strain to survive in the GI tract may be promoted by oligosaccharides facilitating the metabolism and growth of LAB in the lumen (Salminen et al., 1998). Dietary fibre, mainly oligosaccharides and polysaccharides fermented in the colon may act as prebiotics (Fooks et al., 1999; Ziemer and Gibson, 1998). The importance of prebiotics as enhancers of the growth and performance of probiotic bacteria has been documented in humans (Crittenden et al., 2002; Fooks et al., 1999; Van Loo et al., 1999). *Bifidobacterium* sp. and *Lactobacillus* sp. especially produce a positive effect on human health (Gibson and Fuller, 2000; Gmeiner et al., 2000; Schaafsma et al., 1998). The significance of prebiotics in animal diet has also been studied (Hussein et al., 1999) and represents a growing field of research (Gibson and Fuller, 2000).

2.6 Safety of lactic acid bacteria

The use of LAB as a probiotic requires a safety assessment. The functional properties of the strains should be well studied and documented (Holzapfel et al., 2001). Generally recognized health-promoting properties are non-pathogenic behavior, the ability to persist within the GI tract and adhesion, and the ability to

modulate immune responses (Dunne et al., 2001; Gibson and Fuller, 2000; Holzapfel et al., 2001; Reid et al., 2003). Gibson and Fuller (2000) pointed out the importance of considering the possible side effects of probiotics on the consumer, e.g. bloating or blocking the normal functional gut transit. Ishibashi and Yamazaki (2001) pursued the research of bacteria converting food components or biological secretions into secondary substances harmful to the host. Lactobacilli and lactococci commonly hold a GRAS status. Japan legally recognizes functional foods (Foods for Specified Health Use, FOSHU) (Sanders, 2003). Lethal dose (LD_{50}) of LAB was measured for mice by oral administration and found to be > 10^{11} cfu/kg, depending on the strain (Ishibashi and Yamazaki, 2001). The safety of two *Bifidobacterium longum* strains of human origin was evaluated on healthy adult volunteers: no side effects were reported and the immune parameters measured remained without undesirable changes (Mäkeläinen et al., 2001). However, some enterococci such as *E. faecalis* and *E. faecium* are classified in risk group II as pathogens. Special concern has been expressed on the potential risk arising from the existence of antibiotic transferable genes among lactobacilli (Lindgren, 1999). Some species of LAB (*L. acidophilus, L. reuteri, L. rhamnosus, Leuconostoc* spp.) commonly used in the food industry or naturally occuring in raw food materials are resistant to glycopeptide antibiotics such as teicoplanin and vancomycin (Felten et al., 1999; Goldstein et al., 2000; Tynkkynen et al., 1998; Vescovo et al., 1982). Antibiotic resistance encoding genes may transfer into a susceptible strain via a mobile genetic element (Noble et al., 1992; Shlaes et al., 1989), such as plasmids (Leclercq et al., 1987; Teuber et al., 1999; Vescovo et al., 1982) and transposons (Arthur et al., 1993; Hill et al., 1985) to produce new resistant bacterial strains (Danielsen and Wind, 2003). Conjugative transposons are commonly

found in enterococci and streptococci as well as in some *Lactococcus lactis* strains reported to contain a chromosomally located transposon (Rauch and de Vos, 1992). Plasmids of LAB do not commonly carry transmissible antibiotic resistance genes but can take in conjugative transposons and plasmids. Some plasmids, such as those with bacteriocin immunity genes, can integrate into the chromosome (Rauch and de Vos, 1992; Steele and McKay, 1989). Plasmid-linked antibiotic resistance therefore poses a hazard (Lindgren, 1999).

Resistance to glycopeptides in clinical isolates are classified as high-level resistance as well by inducibly and constitutively low-level resistance (Quintiliani et al., 1993). Vancomycin resistance in enterococci is associated with the presence of nucleotide sequences related to *vanA* (Dutka-Malen et al., 1990), *vanB* (Hayden et al., 1993) and *vanC* (Quintiliani et al., 1993). Uses of feeds containing antibiotics and antibiotics for promoting growth in animals, such as fluoroquinolones for poultry, were shown to correlate with antibiotic-resistant bacteria in the animals (Teuber et al., 1999; Witte, 1998). Several *Enterococcus* strains and some of *Lactobacillus* spp. (*L. casei*, *L. plantarum*, *L. rhamnosus*) with transferable vancomycin resistance have been isolated from clinical samples (Cooper et al., 1998; Leclercq et al., 1989; Shlaes et al., 1989), indicating that antibiotic medication may be involved in such cases (Shlaes et al., 1989; Witte, 1998). Lactobacilli appear to be sensitive to penicillins but less so to oxacillin, cefoxitin, ceftriaxone, metronidazole, cephalothin and imipenem (Danielsen and Wind, 2003; Goldstein et al., 2000). Low sensitivity to ampicillin and piperacillin has been fully observed as well (Goldstein et al., 2000). *L. acidophilus* and *L. reuteri* as well as the genus *Enterococcus* are examples of probiotic bacteria

(Benyacoub et al., 2001; Vescovo et al., 1982) resistant to some degree to vancomycin (Arthur et al., 1993; Leclercq et al., 1989; Vescovo et al., 1982).

The antibiotic resistance genes serving as selective markers in LAB have been replaced by food-grade cloning systems (De Vos, 1999) based on i.e., nisin immunity (Takala and Saris, 2002), complementation of deficiency in lactose utilization (Takala et al., 2003), and suppression of nonsense mutation (Sørensen et al., 2000) for positive selection of transformants. The term food-grade can be used when the modified microorganism contains such elements not harming the consumer when present in foods. Food-grade cloning systems need to be based on DNA from LAB or other microbes with a long history of safe use in the food industry (De Vos, 1999). Genetically modified LAB can in future be utilized as improved starters in food fermentation and for the safe production of metabolites used as food additives (De Vos, 1999).

The isolation of LAB from clinical samples has raised debate over the safety of probiotic bacteria and whether or not the bacteria are actually infectious (Adams and Marteau, 1995; Felten et al., 1999; Donohue et al., 1998; Ishibashi and Yamazaki, 2001). Some LAB have been implicated in local systemic infections including septicemia and endocarditis (Antony et al., 1995; Husni et al., 1997; Ishibashi and Yamazaki, 2001; Soleman et al., 2003) as well as liver abscesses (Rautio et al., 1999). In most cases of infection, the organisms were shown to be of host origin. Some cases have been linked to the consumption of probiotics (Salminen et al., 2002; Salminen et al., 2004). Except for enterococci and streptococci, the clinical significance of LAB is low (Boulanger et al., 1991), *L. rhamnosus* being the most frequently isolated LAB from clinical samples (Felten et al. 1999). The isolation of LAB from infections is

likely to be the result of opportunist pathogens on an immunosuppressed host (Ishibashi and Yamazaki, 2001; Salminen et al., 2002). Many factors may promote translocation of intestinal bacteria, such as intestinal mucosal injury, immunodeficiency of the host, an abnormal intestinal bacterial microbiota (Berg, 1995), previous antibiotic treatment, complications from Acquired Immunodeficiency Syndrome (AIDS) and prior hospitalization and surgery (Antony et al., 1995; Cooper et al., 1998; Husni et al., 1997).

The development of novel approaches in food (De Vos et al., 1997; Luoma et al., 2001) and in pharmaceutoclinical therapies (Grangette et al., 2001; Saavedra, 2001; Steidler, 2002) allows broadening the potential for using lactic acid bacteria in food and pharmacology (Kuipers et al., 2000; Mollet, 1999; Renault, 2002). The nature of genetic modifications can be divided into three groups: 1) one-step genetic events like deletions, gene amplifications, plasmid insertions and losses, 2) multi-step genetic rearrangements with DNA of the same species, and 3) trans-species genetic modifications (Mollet, 1999). Kuipers et al. (2000) has emphasized the effective use of gene manipulated LAB in the battle against food spoilage and pathogenic bacteria. As examples, genetically modified LAB have been utilized to improve cheese ripening (Luoma et al., 2001), produce phage resistant starter strains (Moineau, 1999), and protect against tetanus toxin (Grangette et al., 2001) and bovine rotavirus (Enouf et al., 2001). It can be used to treat Shiga toxigenic *Escherichia coli* infections and dysentery in humans (Paton et al., 2000), prevent dental caries (Hillman, 2002) and treat inflammatory bowel disease (Steidler et al., 2003).

Netherwood et al., (1999) studied spontaneous gene transfer in the GI tract and observed that *in vivo* transfer rate in the gut was 0.03 transconjugants per recipient

cell. All new ingredients and genetically modified organisms (GMO) in foods fall under the Novel Foods Regulation of the EU legislation (Feord, 2002; Lindgren, 1999). Renault (2002) discussed the use of genetically engineered LAB in foods, emphasizing the value of risk assessment in correlation with the expected benefits of modified strains. The objective of risk assessment is to identify and evaluate the potential adverse effects of GMOs. The cumulative and long-term effects on human health and the environment have also to be taken into account. Assessment focuses on GM development and the possible genetransfer to host microbiota (Renault, 2002).

2.7 Exploitation of probiotic lactic acid bacteria

The methods for selection of probiotic bacterial strains are discussed in the literature. Host specificity, the generally regarded as safe (GRAS) status, colonization, antimicrobial activity, and desirable metabolic activity are generally agreed upon (Collins et al., 1998; Reid et al., 2003; Tannock, 1998), but issues such as the effect of living versus nonliving probiotics or even their survival in the intestinal tract (Canducci et al., 2000; Reid et al., 2003) remain open. Criteria for quality, including the sensory characteristics of probiotic strains, is well established (O'Sullivan et al., 2002; Reid et al., 2003) as are those for technological suitability (Charteris et al., 1998a; Knorr, 1998). In addition to *in vitro* experiments (Charteris et al., 1998b, Gibson and Fuller, 2000), animal models (Borriello, 1990; Cross, 2002; Mallett et al., 1987) and GI tract simulation studies (Gmeiner, et al., 2000) have been employed for probiotic detection. The ultimate test for probiotic functionality is a double blind, placebo-controlled and randomised human study (Gibson and Fuller, 2000). Uses of probiotic LAB are listed in Table 2. Prebiotic and probiotic based biotherapy has

shown potential as an alternative for medical treatment (Dunne and Shanahan, 2003). The demonstration of probiotic activity of a given strain requires a well - designed, double blind, placebo-controlled host-specific study also showing resistance to technological processes, meaning viability and activity throughout processing phases (Dunne et al., 2001). Each potential probiotic strain must be documented independently, without extrapolating any data from closely related strains and employing only well defined strains, products and study populations in trials. Results should be confirmed by independent research groups and published in a peer-reviewed journal (Berg, 1998; Salminen et al., 1996; Salminen et al., 1998).

2.8 Researches for improving probiotic products

Many researchers have reported on techniques to improve probiotic products. Larisch et al. (1994) revealed to microencapsulate *Lactobacillus lactis* subsp. cremoris within alginate/poly-L-lysine (alg/PLL), nylon or cross-liked polyethyleneimine (PEI) membranes. Toxic effects were observed with solvents and reagents used in nylon and PEI membrane formation. Alg/PLL encapsulation resulted in viable and active cell preparations which acidified milk at a rate propotional to the cell concentration, but at rates less than of free cell preparations. At 4 x 10^8 colony-forming units (cfu/ml milk), encapsulated cells took 17 percent longer than free lactococci to reduce the pH of milk to 5.5. Similar activities of free and microencapsulated cells may be attained at higher cell concentrations (10^9 cfu/ml milk).

Maggi et al. (2000) evaluated ten strains of lactobacilli for the administration of viable microorganisms to restore the normal indigenous flora in the treatment of

urogenital tract infections in women. The microorganisms were formulated in single – and double-layer vaginal tablets. Their results showed that three out of ten strains appear particularly suitable for their application in the treatment of urogenital tract infections.

O'Riordan et al. (2001) optimized a spray coating process for the production of encapsulated microspheres containing viable *Bifidobacterium* cells. They also determined whether the readily gelatinized modified starch coating used in this study improved bacterial survival in foods under acid conditions. The results showed that the optimal spray drying conditions were using inlet temperature of 100 ^{o}C and a feed rate of 5 ml/min. The average size of *Bifidobacterium* PL1-containing starch microparticles was determined by scanning electron microscopy to be of the order of 5 μm. The starch-coated cells did not display any enhanced viability compared with free PL1 cells when exposed to acid conditions for 6 hours or in two dry food preparations over 20 days storage at 19-24^{o}C.

Chan and Zhang (2001) evaluated the potential use to compression coating as an alternative method to encapsulate probiotic bacteria *Lactobacillus acidophilus* for improving their storage stability. They found that when the cell powder were compressed, there was little loss of viability of the bacteria at pressures up to 120 MPa, beyond which the cell death was more significant, but the degree of cell injures seemed to increase with the compression pressure gradually. When the compression pressure used was at 60 MPa, the stability of the encapsulated cells was about 10 times higher than free cell powders and cell pellets after 30 days storage at 25 ^{o}C

Wiwat et *al.* (2001) characterized certain properties of Thailand local isolates of lactobacillus, *Lactobacillus acidophilus* 51, *L.casei* 14 and *L.fermentum* 71, and

observed their survival during storage in formulated capsules. They found that each strain could tolerate 1% bile salt, adhere well to the smooth surface of aluminium wire and inhibit the growth of *Escheriichia coli, Staphylococcus aureus, Bacillus cereus* and *Salmonella typhimurium.* They were not toxic to mice in an acute toxicity test. A comparative study of the efficiency of different suspension media demonstrated that 20% (skimmed milk) + 1% (glutamate) gave the highest survival rate after freeze-drying. The capsule were prepared using freeze-dried cultures, carrot powder and Aerosil®. The stability test was done by survival count monthly for 11 months, from the same container, kept tightly closed at 4 ^{o}C. Loss of cell viability was observed each month; the rate was first order. Other physical properties, weight uniformity and disintegration time, remain more or less the same.

Picot and Lacroix (2003) investigated the use of micronization to prepare a freeze-dried powder of three strains of probiotic bacteria (*Bifidobacterium* sp.) with suitable particle-size distribution for the production of low-diameter multiphase spray-dried microcapsules. In conclusion, the researchers suggested that micronization is an effective way to reduce powder particle size of freeze-dried cultures with an acceptable mortality rate before producing microcapsules with low diameters for protection of sensitive probiotic bacteria.

Stadler and Viernstein (2003) reported the optimization of a formulation containing viable lactic acid bacteria (LAB). Tablets formulations of LAB were developed, using hydroxypropylmethylcellulose acetate succinate (HPMCAS), sodium alginate, calcium alginate, pectin and Metolose®, as matrix forming components. Tablets were modified with respect to LAB content, amount of applied excipient per tablet, and compaction forces. It was found that the best protective

qualities against artificial gastric juice were observed when tablets were prepared from compaction mixtures of LAB, HPMCAS and sodium alginate.

2.9 Tablets

Compared to other oral dosage forms tablets are the manufacturer's dosage form of choice because of their relatively low cost of manufacture, package, and shipment.

Advantages of tablets as a dosage form (Conway, 2008)

- Easy to handle
- Variety of manufacturing methods
- Can be mass produced at low cost
- Consistent quality and dosing precision
- Can be self - administered
- Enhanced mechanical, chemical, and microbiological stability compared to liquid dosage forms
- Tamperproof
- Lend themselves to adaptation for other profiles, e.g., coating for sustained release

Pharmaceutical compressed tables are prepared by placing an appropriate powder mix, organulation, in a metal die on a tablet press. At the base of the die is a lower punch, and above the die is an upper punch. When the upper punch is forced down on the powder mix (single station press) or when the upper punches squeeze together (rotary or multiple station press), the powder is forced into a tablet (Kottke and Rudnic, 2002).

Compacted or compressed tablets are produced from granulations or powder mixtures made by the following general techniques:

- Direct compression (dry mixing and blending)

-Wet granulation (high shear, low shear) combined with tray drying or fluid-bed drying

-Wet granulation and drying in the same equipment

- Dry granulation by roller compaction or slugging

Over the past four decades, improvements in the availability of excipients with consistent physical properties (including particle size and shape, and improved functionality such as compaction and flow), have revolutionized tablet production on a commercial scale. In addition, the availability of a diversity of equipment for the wet granulation process (including high-shear granulators, fluid-bed granulators and dryers, extrusion granulators, continuous granulators, and granulators with wet granulation and drying combined in the same equipment), have made tablet production more economical. However, tablet production by direct compression still remains the method of choice because it offers economic advantages by eliminating the wet granulation and drying steps. A summary of the general advantages and dis advantages of direct compression have been outlined in Table

Specialized processes may be used for certain types of tablets, such as extrusion; a combination of extrusion, spheronization, and compaction; a coating for modified-release tablets; and freeze drying for prompt-release tablets.

Table 1 Advantages and disadvantages of direct compression (Jivraj et al., 2000)

Advantages	Disadvantages
- Requires fewer unit operations compared with wet granulation (shorter processing time and lower energy consumption)	- Issues with segregation –these can be reduced by matching the particle size and density of the active drug substance with excipient
- Fewer stability issues for actives that are sensitive to heat or moisture	- In general, the drug content is limited to approximately 305 or approximately 50 mg
- For certain compounds, faster dissolution rates maybe generated from tablets prepared by direct compression compared with wet granulation	- May not be applicable for material processing a low bulk density because after compression the tablets produced may be too thin
- Fewer excipients may be needed in a direct compression formula	- Not suited for poorly flowing drug compounds
	Statistic charges may develop on the drug particles or excipients during mixing, which which may lead to agglomeration of particles producing poor mixing

2.10 Polymers in colon-specific drug delivery

The interest for polymers as pharmaceutical excipients and carriers is continuously growing. In this context, colon-specific drug delivery systems formulated with enteric polymers have received considerable attention. Their role is to

protect the active agent from the acidic medium of the stomach and to deliver it to the mucosal intestinal site.

2.10.1 Hydroxypropyl methylcellulose (HPMC)

Hydroxypropylmethylcellulose (HPMC) is one of the most widely used pharmaceutical aids as a binder for tableting, and could have the dual functions as an effect of plasma irradiation due to the presence of both structural features of hydroxypropylcellulose (HPC) and methylcellulose (MC). In oral products, HPMC is primarily used as a tablet binder, in film coating and as an extended release tablet matrix. There are several derivatives which used as enteric coating agent such as hydroxypropylmethylcellulose acetate succinate (HPMCAS) and hydroxypropylmethylcellulose phthalate (HPMCP).

2.10.2 Hydroxypropylmethylcellulose phthalate (HPMCP)

Hydroxypropylmethylcellulose phthalate (HPMCP) is prepared from hydroxypropylmethylcellulose (HPMC) by esterification with phthalic anhydride resulting in a basic repeating structure where the hydroxyl groups of the glucose unit are substituted by methoxyl, hydroxypropyl and carboxybenzyl groups. The chemical structure of HPMCP was shown in **Figure 1**. The threshold pH valid disintergration of HPMCP can be controlled by varying the phthalyl content. It is insoluble in gastric fluid (pH~1.5), and thus provides protection against dissolution of the drug contained within it. It is not until the dosage form presents within the upper small intestine where there is a shift to pH~5.5 that HPMCP undergoes rapid dissolution, thus releasing the active pharmaceutical ingredient. HPMCP (also known as hypromellose

phthalate) is widely used in so called enteric systems as a coating material for tablets and granules. There are two types of HPMCP with different solubility. HP-55 and HP-50, are available.

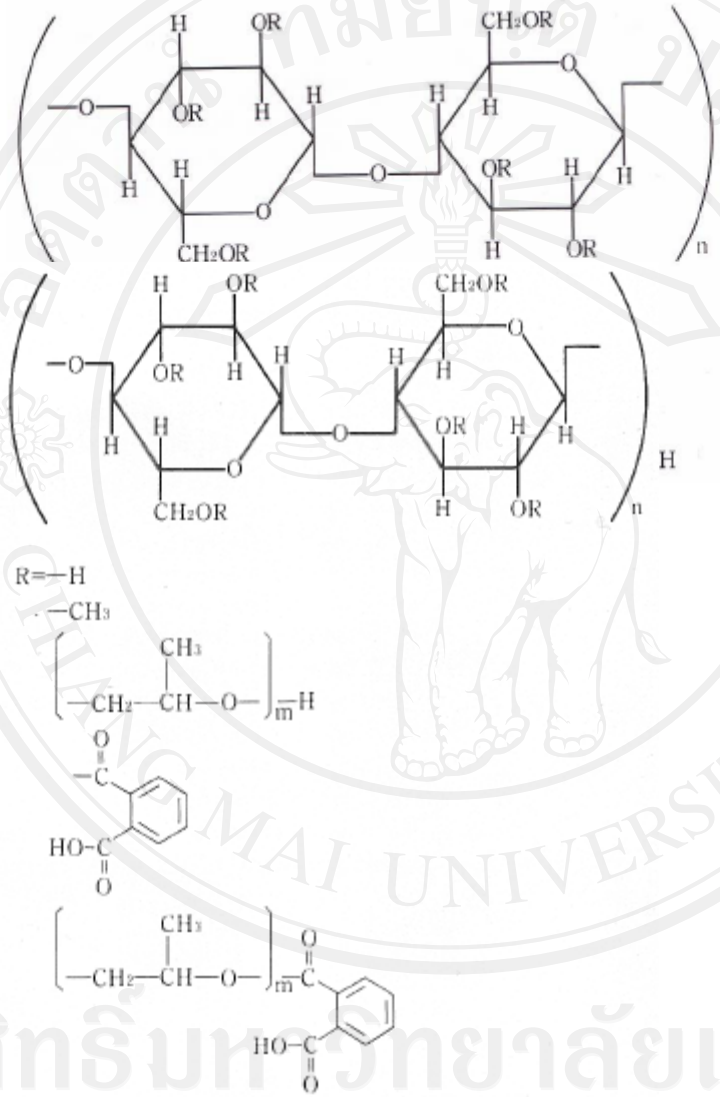

Figure 1 Structure of hydroxypropyl methylcellulose phthalate (HPMCP)

2.10.3 Pectins

Pectins are non-starch, linear polysaccharides extracted from the plant cell walls. They are predominantly linear polymers of mainly α-(1-4)- linked D-galacturonic acid residues interrupted by 1,2- linked L-rhamnose residues. Pectin has

a few hundred to about one thousand building blocks per molecule, corresponding to an average molecular weight of about 50,000 to about 180,000 (**Figure 2**). These polysaccharides remain intact in the physiological environment of the stomach and the small intestine, but are degraded by the bacterial inhabitants of the human colon (Werch and Ivy, 1941; Salyers et al., 1977). Being soluble in water, pectin is not able to shield its drug load effectively during its passage through the stomach and small intestine. It was found that a coat of a considerable thickness was required to protect the drug core in simulated in vivo conditions (Ashford et al., 1993). So, the focus shifted to the development of such derivatives of pectin which were less water soluble but were degradable by the colonic microflora (Rubinstein et al., 1993).

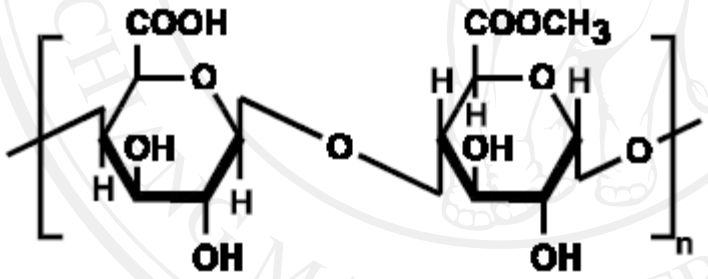

Figure 2 Structure of pectin

2.10.4 Inulin

Inulin is a naturally occurring polysaccharide (Van Loo et al., 1995) found in many plants, such as onion, garlic, chicory, artichoke. Chemically, it consists of β- 2-1 linked D-fructose molecules, having a glucosyl unit at the reducing end (Roberfroid, 1993) (**Figure 3**). Chicory inulin currently used in the food industry (De Bruyn et al. 1992). It is not hydrolysed by the secretions of the human digestive tract (Dysseler and Hoffem, 1995). Inulin has attracted much attention recently as nonabsorbable

carbohydrate with prebiotic properties, decrease fecal odor components, reduce blood cholesterol, prevent or inhibit the occurrence of some types of cancer, enhance vitamin synthesis, increase mineral absorption, and stimulate the immune system (Jenkins et al., 1999). Bacteria present in the colon especially *Bifidobacteria*, which constitute up to 25% of the normal gut flora in man (McKellar and Modler, 1989) are known to ferment inulin (Wang and Gibson, 1993; Gibson and Roberfroid, 1995). To overcome the poor film forming property and to control the swelling of inulins, they have been evaluated for colon-targeting in combination with synthetic film forming polymers. The mixed films thus prepared resisted degradation in the upper GIT and fermented in the colon by *Bifidobacteria* and *Bacteroides*. Vervoort and Kinget (1996) incorporated highly polymerised inulin in Eudragit RS films which were degraded in human fecal medium. Also, the permeability of these membranes increased significantly after incubation in the fecal medium.

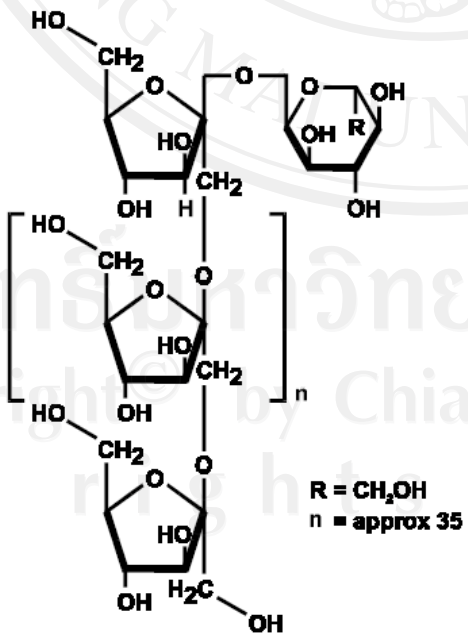

Figure 3 Structure of inulin

2.10.5 Alginates

Alginates are a linear polymer which have 1-4'linked-β-D-mannuronic acid and α-L-guluronic acid residues arranged as blocks of either type of unit or as a random distribution of each type (**Figure 4**). Alginates do not gel since they have poly(L-gluronic acids) which are rigid, Ca^{++} ions induce gelation (Sinha and Kumria, 2001).

Figure 4 Structure of alginate

2.11 Banana

Bananas (*Musa* spp.) is the world's most widely consumed fruit. According to the United Nations Food and Agriculture Organization, bananas are the world's fourth most important food corp. In the Asia-Pacific region, bananas are the most widely produced fruit in Philippines, Thailand, Indonesia, and India. In Thailand, both unprocessed bananas and their processed products are generally consumed. Ripe bananas are not only cheap but also claimed with its health benefits (Kruawan et al., 2004). In Thailand, there are many cultivars of bananas (Thai word for banana is Kluai), the most common are: Kluai Hom, Kluai Nam Wa, Kluai Khai, and Kluai Hakmuk. Pannangpetch et al. (2001) found that the extract of raw Hom (*Musa sapientum* Linn.) and Palo (*Musa paradisiaca*) bananas protected the stomach from the indomethacin-induced injuries. They also presented that different kinds of banana

have varying gastroprotective qualities. Whereas, Mukhopadhyaya and co-researchers (1987) found that the banana powder (*Musa sapientum* Linn.var.*paradiscica*) treatment was observed to strengthen gastric mucosal resistance in rats. Moreover, pharmacological studies showed that bananas had antifungal (Ranasinghe et al., 2002) and antibacterial effects (Ono et al., 1998). It is also very interesting to explore other benefits of ripe bananas, a cheap fruit commonly eaten by Thai people.