

**Isolation, identification and exploitation of lactic acid bacteria from human
and animal microbiota**

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Publications I - IV	

List of original publications

This thesis is based on the following original papers, referred to in the text by their Roman numerals, as well as on unpublished results.

- I Beasley, S.S., P.E.J. Saris. 2004.
Nisin-producing *Lactococcus lactis* strains isolated from human milk
Applied and Environmental Microbiology 70: 5051-5053.
- II Beasley, S., H. Tuorila, P. Saris. 2003.
Fermented soymilk with a monoculture of *Lactococcus lactis*.
International Journal of Food Microbiology 81: 159-162.
- III Beasley, S.S., T.M. Takala, J. Reunanen, J. Apajalahti, P.E.J. Saris. 2004.
Characterization and electrotransformation of *Lactobacillus crispatus* from chicken
crop and intestine.
Poultry Science 83: 45-48.
- IV Beasley, S.S., P.E.J. Saris. 2004.
Lactic acid bacteria isolated from canine faeces
Veterinary Microbiology, manuscript

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Author's contribution

Publication I

Carried out the experimental work (excluding the riboprinter analysis and the cloning of the strain *Lactococcus lactis* LL3), interpreted the results and wrote the Paper in collaboration with the corresponding author.

Publication II

Performed the experimental work, interpreted the results (excluding sensory characterization) and wrote the Paper in conjunction with the coauthors.

Publication III

Conducted the main part of the experimental work (excluding fatty acid analysis and dendrogram based on partial 16S rRNA sequencing results), interpreted the results and wrote the Paper in conjunction with T. Takala and the corresponding author.

Publication IV

Designed the experiments, interpreted the results, wrote the Paper and acted as the corresponding author.

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bp	Base pair
CE	Competitive exclusion
CFU	Colony-forming unit
DGGE	Denaturing gradient gel electrophoresis
ED	Euclidian distance
EMBL	European Molecular Biology Laboratory
ERM	Erythromycin
FDA	Food and Drug Administration
FOSHU	Foods for Specified Health Use
GFP	Green fluorescent protein
GI	Gastrointestinal
GMO	Genetically modified organism
GRAS	Generally Recognized As Safe
IDF	International Dairy Federation
IG	Immunoglobulin
IU	International unit
Kb	Kilobase
LAB	Lactic acid bacteria
LB	Luria Bertani medium
LBS	<i>Lactobacillus</i> selective medium
LD ₅₀	Lethal dose killing 50% of the tested subjects
LDL	Low density lipoprotein
mLBS	modified <i>Lactobacillus</i> selective medium (acetic acid omitted)
MRS	de Man, Rogosa and Sharpe medium
NCBI	National Center for Biotechnology Information
Nd	Not determined
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescent unit
RLF	Reconstituted lactobacilli-free (mouse)
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
TGGE	Temperature gradient gel electrophoresis
TSA	Trypticase Soy agar
UHT	Ultra high temperature
UPGMA	Unweighted pair-group mean arithmetic method
VRB	Violet red bile medium
YGC	Yeast glucose chloramphenicol medium

1. Introduction

1.1. 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; Jay, 2000; Holzapfel et al., 2001; 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 comparative 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).

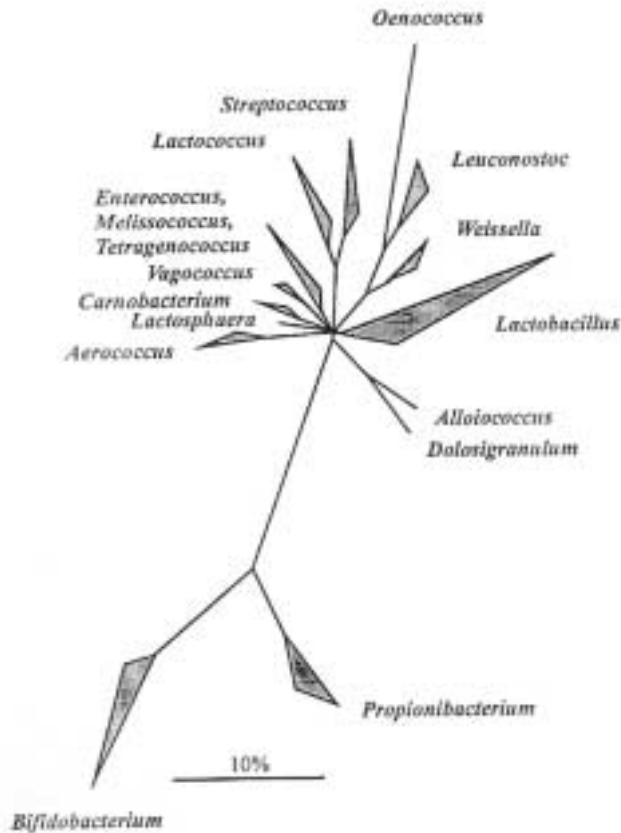


Fig 1. Consensus tree based on comparative analysis of the 16S rRNA gene, showing the major phylogenetic groups of lactic acid bacteria with low mol% of guanine plus cytosine in the DNA and the nonrelated Gram-positive genera *Bifidobacterium* and *Propionibacterium* (Holzapfel et al., 2001).

LAB were first isolated from milk (Carr et al., 2002; Metchnikoff, 1908; 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, 2001; 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; Rodrigues et al., 2003), some of which may act as antimicrobials.

1.2. Lactic acid bacteria benefiting health

LAB have been cited to be part of human (Fuller, 1991; Goldin, 1990; Holzapfel et al., 2001; Reid, 2001; Schrezenmeir and de Vrese, 2001; Sghir et al., 2000) and animal (Batt et al., 1991; Benno et al., 1992; Fujisawa and Mitsuoka, 1996; Perdigon et al., 2001; Rodríguez et al., 2003; Schrezenmeir and de Vrese, 2001) microbiota. The neonates receive their microbiota primarily in labor and later from the environment (Edwards and Parrett, 2002; Fuller, 1989; Fuller and Gibson, 1998; Metchnikoff, 1908). LAB and bifidobacteria dominate the microbiota of the full-term neonate (Hall et al., 1990), especially when breast-fed (Edwards and Parrett, 2002; Lönnerdal, 2000) with a health-promoting effect on the child (Arici et al., 2004; Boris et al., 1998; Edwards and Parrett, 2002). Heikkilä and Saris (2003) isolated LAB from human milk. Martín et al. (2003) detected *Lactobacillus gasseri* from breast-feeding mothers and children in pair and observed coccoid LAB sharing identical randomly amplified polymorphic DNA (RAPD) patterns. Although it is difficult for microbes to establish themselves in an already colonized ecosystem (Tannock, 1990), the health impact of microbiota consisting of LAB is well documented in humans (Bezkorovainy, 2001; Fooks et al., 1999; Majamaa and Isolauri, 1997; Reid et al., 2003) and in animals (Bezkorovainy, 2001; Ehrmann et al., 2002; Fujisawa and Mitsuoka, 1996; Nurmi and Rantala, 1973). Gut bacteria are anticipated to interact with the host, encompassing direct interaction between bacteria and host epithelial cells (de Vos et al., 2004).

LAB are regarded as a major group of probiotic bacteria (Collins et al., 1998; Metchnikoff, 1908; Schrezenmeir and de Vrese, 2001; Tannock, 1998). 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. Several lactobacilli, lactococci and bifidobacteria are held to be health-benefiting bacteria (Rolfe, 2000; Tuohy et al., 2003), but little is known about the probiotic mechanisms of gut microbiota (Gibson and Fuller, 2000). LAB constitute an integral part of the healthy gastrointestinal (GI) microecology and are involved in the host metabolism (Fernandes et al., 1987). Fermentation has been specified as a mechanism of probiotics (Gibson and Fuller, 2000; Metchnikoff, 1908). LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into

short-chain fatty acids and other organic acids and gases (Gibson and Fuller, 2000; Gorbach, 1990; Jay, 2000). LAB synthesize enzymes, vitamins, antioxidants and bacteriocins (Fernandes et al., 1987; Knorr, 1998). With these properties, intestinal LAB constitute an important mechanism for the metabolism and detoxification of foreign substances entering the body (Salminen, 1990). The health-promoting effects of LAB are strain specific and result in different mechanisms to produce beneficial health impacts (Table 1).

LAB have been found to control intestinal disorders, partially due to serum antibodies IgG, and secretory IgA and IgM enhancing immune response (Cross, 2002; Grangette et al., 2001; Kimura et al., 1997; Link-Amster et al., 1994; Perdigón et al., 1999). Certain strains of LAB can intermittently translocate across the intestinal mucosa without causing infection (Berg, 1995), thus influencing systemic immune events (Cross, 2002). Evidence has been presented that some lactobacilli can directly stimulate the immune system on the gut mucosal surface via localized GI tract lymphoid cell foci (Perdigón et al., 1999). Morishita et al. (1971) demonstrated that intestinal origin LAB established in the digestive tract of germ-free chickens better than did non-intestinal LAB strains. Several reports have been made on LAB surviving the GI tract of humans and animals (Drouault et al., 1999; Klijn et al., 1995; Yuki et al., 1999). A number of mechanisms work to prevent harmful bacteria from growing on and attaching to the intestinal epithelium: production and secretion of antimicrobial agents such as bacteriocins and organic acids (Fooks et al., 1999; Reid, 2001), adherence via competition for the binding sites and steric hindrance (Bezkorovainy, 2001; Boris et al., 1998; Schrezenmeir and de Vrese, 2001) and barriers interfering with pathogens and hence promoting the elimination of harmful bacteria (Boris et al., 1998). Boris et al (1998) reported vaginal LAB strains being able to self-aggregate in a process mediated by surface proteins or lipoproteins, depending on the strain. In addition, strains adhered to vaginal epithelial cells, interfered with other bacteria and coaggregated with tested pathogens *in vitro*. Both aggregation and adhesion may favor the vaginal epithelium through the formation of a bacterial film contributing to the exclusion of pathogens from the vaginal mucosa (Boris et al., 1998). *L. rhamnosus* strain GG and *L. reuteri* ING1 have been shown to exhibit disease-specific adhesion to intestinal tissue (Ouweland et al., 2003). Additionally, reports have been published on bacteriocin production by some probiotic bacteria targeting pathogenic bacteria *in vitro* (Elliason and Tatini, 1999; O'Sullivan et al., 2002; Ziemer and Gibson, 1998). Reutericyclin, an antibiotic produced by *Lactobacillus reuteri* LTH2584, has recently been discovered to inhibit a broad range of bacteria (Gänzle et al., 2000). Its biological activity is comparable to that of nisin. The colonized *L. reuteri* LTH2584 cells were recovered from the intestine of reconstituted lactobacilli-free (RLF) mice in high cell counts. This strain is proposed to be a valuable tool for studying the role of antibacterial agents in intestinal habitats. (Gänzle, 2004).

Table 1. Selected health-promoting lactic acid bacteria, their impacts and mechanisms.

Health effect	Mechanisms	Strain example	Reference
Relieve lactose intolerance symptoms	Hydrolysing lactose into glucose and galactose and forming the physical appearance of milk into a thick substance, such as yogurt, that passes through the GI tract slowly, reducing the lactose pulse in the colon.	<i>Lactobacillus rhamnosus</i> GG	Drouault and Corthier, 2001 Heyman, 2000 Hove et al., 1999
Control viral, bacterial and antibiotic-associated diarrhea in humans and animals	Reinforcing the local immune defence through specific IgA response to rotavirus and pathogens.	<i>L. rhamnosus</i> GG <i>L. reuterii</i> <i>Enterococcus faecium</i>	Ehrmann et al., 2002 Heyman, 2000 Majamaa and Isolauri, 1997 Oksanen et al., 1990 Vahjen and Männer, 2003
Prevention of allergies and atopic eczema	Prevention is partially due to serum antibodies IgG and secretory IgA and IgM immune response enhanced by probiotics.	<i>L. rhamnosus</i> GG <i>Bifidobacterium lactis</i> Bb-12	Cross, 2002 Link-Amster et al., 1994 Perdigón et al., 1999 Majamaa and Isolauri, 1997 Isolauri et al., 2000
Prevention of intestinal bacterial enzymes involved in the synthesis of colonic carcinogens	Enhancing host's immune response, binding and degrading carcinogens, producing antimutagenic compounds, alteration of metabolic activities of intestinal bacteria and alteration of physiochemical conditions in colon might work to prevent cancer.	<i>B. bifidum</i> <i>B. infantis</i> <i>B. longum</i> <i>L. acidophilus</i> <i>L. paracasei</i>	Hirayama and Rafter, 2000 Rolfe, 2000 Sanders, 1998
Inactivation and reduction of pathogenic bacteria	Production of antimicrobial substances and Competitive Exclusion (CE).	<i>Lactococcus lactis</i> <i>Pediococcus acidilactici</i>	Elliason and Tatini, 1999 Nurmi and Rantala, 1973 O'Sullivan et al., 2002
Direct stimulation of the immune system on the gut mucosal surface	Adherence to mammalian extracellular matrix. Stimulation via localized GI tract lymphoid cell foci.	<i>L. crispatus</i> strains JCM1132, ST1, A33 and 134mi <i>L. gasseri</i> CT5 <i>L. reuteri</i> CT7	Edelman et al., 2002 Toba et al., 1995

However, the validity of the probiotic concept has been questioned (Shanahan, 2003; Tannock, 2003). The adequate information by which the consumer and health professional can judge the efficacy and safety of retail probiotics is lacking. Probiotic products have not been subjected to large scale trials of efficacy that are used in the pharmaceutical industry. (Tannock, 2003). As an example, no direct evidence of LAB suppressing colon cancer has yet been put forward (Hove et al., 1999). In addition, the difficulty of *in vivo* studies poses problems in further showing the complete effects of probiotics (Shanahan, 2003). Much remains to be done to understand the full effect of probiotics, given the extreme complexity of the biological systems of humans and their interactivity (Klaenhammer and Kullen, 1999). Studies conducted on bacteria beneficial to health covers only a segment of the human ecosystem (Klaenhammer and Kullen, 1999; Shanahan, 2003). *In vivo* sampling in humans also raises ethical issues limiting the scope of physiological and clinical testing. For this reason, alternative methods are implied, such as performing mucosal biopsies on specific parts of the GI tract (Shanahan, 2003) and detecting selective bacteria from faecal samples (Simmering and Blaut, 2001). The stability of probiotic LAB in the GI tract is another concern. Heilig et al (2002) showed that GI tract bacterial biota altered during first five months of an infant's life, while the composition of the *Lactobacillus* community remained more stable over a two-year study on adults, with individual differences. *L. lactis* may survive in human (Klijn et al., 1995) and in mouse (Drouault et al., 1999) GI tract.

1.2.1. Bacteriocins produced by lactic acid bacteria

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), non-lantibiotics, small heat-stable peptides (Class II) and large heat-labile protein (Class III) (O'Sullivan, et al., 2002). The lantibiotic nisin naturally produced by *Lactococcus lactis* ssp. *lactis* is commercially available as food additive E234 (Anonymous, 1994). The nisin variants A and Z, differing by one amino acid (de Vos et al., 1993), are approved for use in foodstuffs by food additive legislating bodies in the US (Food and Drug Administration, FDA) and in the EU (Thomas et al., 2000). In addition, a new nisin variant, nisin Q, has been isolated from a *L. lactis* strain found in river water in Japan. Nisin Q differs in four amino acids as a mature peptide and in two amino acids of the leader sequence. (Zendo et al., 2003). All forms of nisin are antimicrobially active against Gram-positive bacteria, such as LAB, *Listeria* sp., *Micrococcus* sp. and sporeforming bacteria like *Bacillus* sp. and *Clostridium* sp. (McAuliffe et al., 2001; Thomas et al., 2000; Zendo et al., 2003). The inhibiting mode of nisin towards vegetative cells consists of several phases. Nisin accumulates on the cell membrane and inserts into it, then aggregates within the membrane to form a water-filled pore (Nissen-Meyer et al., 1992;

Thomas et al., 2000; McAuliffe et al., 2001). Another model suggests that nisin molecules bind by electrostatic interactions to the anionic membrane surface, leading to a high local concentration that disturbs the lipid dynamics and causes localized strains, forcing the nisin into the membrane (Driessen et al., 1995). At this stage, a voltage-dependent pore is formed leading to the dissipation of the bacterial proton motive force. Loss of the proton motive force, required for ATP synthesis and the transport of ions, causes cell death through depletion of energy dependent reactions (Breukink and de Kruijff, 1999). Nisin is also known to inhibit peptidoglycan biosynthesis by interacting with cell wall precursors, lipid I and lipid II (Wiedemann et al., 2004). Breukink et al. (2003) concluded that nisin-lipid II interaction stabilized the pore complex. The electric transmembrane potential is strongly reduced in the presence of nisin and lipid II (Wiedemann et al., 2004). Furthermore, nisin inactivates endospores by preventing post-germination swelling and subsequent spore outgrowth (Hitchins et al., 1963; Thomas et al., 2000).

LAB capable of secreting antimicrobial peptides are used in a probiotic manner as food preservatives as well as health-promoting agents for humans (Barefoot and Nettles, 1993; Ryan et al., 1996; Ocaña et al., 1999; O'Sullivan et al., 2002) and animals (Robredo and Torres, 2000; Ryan et al., 1996). Nisin applied as a food preservative extends the shelf life of a product (O'Sullivan, et al., 2002; Zottola et al., 1994). It is relatively stable in foodstuffs since 15 – 20% of nisin is lost in heat treatment (Thomas et al., 2000). For probiotic purposes, bacteriocins are generally produced by a LAB strain in the product (Bernet-Camard et al., 1997; Dunne and Shanahan, 2003; Joosten and Nuñez, 1996; Yuki et al., 1999). The bacteriocin concentration then remains lower than when the purified antimicrobial agent is added.

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 spore-forming 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 heteropolysaccharides, whereas Mantovani and Russell (2001) reported nisin-resistant *S. bovis* JB1 cells having more lipoteichoic acid than nisin-sensitive cells.

1.2.2. Effect of prebiotics on probiotic bacteria

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., 1998a). 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).

1.3. 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., 1986) 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., 1998b).

Table 2. The probiotic effect of lactic acid bacteria (LAB) in human and animal health.

Medical target	Example strain	Reference
Prevent food allergy	<i>L. rhamnosus</i> GG	Sütas et al., 1996
Block formation of biogenic amines	<i>L. lactis</i> ESI 561 <i>E. faecalis</i> INIA 4-07 <i>E. faecalis</i> EFS 2	Joosten et al., 1996
Overcome lactose intolerance	<i>L. acidophilus</i>	Gilliland and Kim, 1984
Prevent diarrhea (antibiotic-induced, rotavirus, travellers, community acquired, <i>Clostridium difficile</i> colitis)	LAB <i>L. rhamnosus</i> GG <i>L. acidophilus</i> LB	Fooks et al., 1999 Heyman, 2000 Oksanen et al., 1990 Simakachorn et al., 2000 Sanders, 2003
Reduce intestinal disorders and pouchitis	LAB <i>L. rhamnosus</i> GG	Gionchetti et al., 2000 Kuisma et al., 2003
Suppress side effects of <i>Helicobacter pylori</i> medication with antibiotics.	<i>L. acidophilus</i>	Canducci et al., 2000
Treat Crohn's disease, ulcerative colitis and inflammatory bowel disease (IBD)	<i>L. rhamnosus</i> GG <i>B. infantis</i> UCC35624 LAB	Gupta et al., 2000 Von Wright et al., 2002 Marteau et al., 2002
Stimulate anticarcinogenic activity	LAB <i>L. acidophilus</i>	Goldin, 1990 Hirayma and Rafter, 2000
Treat coronary heart disease and anticholesterolaemic effects	<i>L. acidophilus</i>	Schaafsma et al., 1998 Gilliland et al., 1985
Control of human urinary tract infection and vaginosis	<i>L. rhamnosus</i> GG <i>L. rhamnosus</i> GR-1	Kontiokari et al., 2001 Reid, 2001 Reid, 2002
Prevent kidney stones	<i>L. acidophilus</i> <i>L. plantarum</i> <i>L. brevis</i> <i>S. thermophilus</i> <i>B. infantis</i>	Campieri et al., 2001
Treat atopic disease	<i>L. rhamnosus</i> GG	Kalliomäki et al., 2001
Prevent caries formation	<i>L. rhamnosus</i> GG	Näse et al., 2001
Protection against tetanus toxin	<i>L. plantarum</i>	Grangette et al., 2001
Treat chronic fatigue syndrome	LAB	Logan et al., 2003
Inhibit pathogens causing bovine mastitis	<i>L. lactis</i> DPC3147	Ryan et al., 1998
Feed supplement for growth promotion in animals	<i>L. brevis</i> C10	Jin et al., 1998
Reduce pathogens in chickens by Competitive Exclusion (CE)	Undefined faecal cultures	Nurmi and Rantala, 1973
Inhibit enteropathogens in small intestine of animals	<i>L. acidophilus</i> LA1	Bernet-Camard et al., 1997

LAB = Lactic acid bacteria species not specified.

1.4. 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 recognises functional foods (Foods for Specified Health Use, FOSHU) (Sanders, 2003). Lethal dose (LD₅₀) of LAB was measured for mice by oral administration and found to be > 10¹¹ 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 (Anonymous, 2004c). 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 occurring 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 (Immonen et al., 1998; 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). Use 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, ceftioxin, 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.a., 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., 1996; 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) allow 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). No GMO has yet been authorized as a feed additive in Europe (Anonymous, 2001). 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 gene-transfer to host microbiota. (Renault, 2002).

Since the LAB of human and animal microbiota is regarded safe and is believed to be beneficial to the host, this study aims at isolating LAB from human and animal microbiota and screening them for potential probiotic use.

2. Aims of the study

Humans and animals carry a specific microbiota consisting in part of lactic acid bacteria. These LAB are believed to be beneficial to the host. The probiotic effect has been widely researched in humans and in rat models. The present study aims at isolating and characterizing LAB from human and animal microbiota and charting the possible exploitation of such bacteria. The steps taken to reach this objective consist of:

1. Isolation of antagonistic bacteria from human milk.
2. Determining the potential of human-origin LAB for human protective use.
3. Selection of electrotransformable LAB from chicken intestine to improve feed absorption and chicken health.
4. Isolation and identification of LAB from canine faeces. Screening for probiotic strains for canine health benefits.

3. Materials and methods

3.1. Strains and plasmids

Table 3. Strains used in this study.

Strain	Studied in Paper nr	Strain origin and reference
<i>Bacillus licheniformis</i> 553/1		HAMBI, Salkinoja-Salonen et al., 1999
<i>Escherichia coli</i> TG-1	III	Genesit Ltd, Sambrook et al., 1989a
<i>Lactobacillus crispatus</i> 28mc	III	This work
<i>Lactobacillus crispatus</i> 145mc	III	This work
<i>Lactobacillus crispatus</i> 29mi	III	This work
<i>Lactobacillus crispatus</i> 81mi	III	This work
<i>Lactobacillus crispatus</i> 101mi	III	This work
<i>Lactobacillus crispatus</i> 119mi	III	This work
<i>Lactobacillus crispatus</i> 134mi	III	Danisco, Edelman et al., 2002
<i>Lactobacillus crispatus</i> A33	III	Danisco, Edelman et al., 2002
<i>Lactobacillus crispatus</i> ATCC33820	III	ATCC, Skerman et al., 1980
<i>Lactobacillus fermentum</i> LAB8	IV	This work
<i>Lactobacillus mucosae</i> LAB12	IV	This work
<i>Lactobacillus rhamnosus</i> LAB11	IV	This work
<i>Lactobacillus salivarius</i> LAB9	IV	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> LL3	I, II	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 310	I	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 2410	I	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 3A	I	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 4B	I	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 6A	I	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 6B	I	This work
<i>Lactococcus lactis</i> ssp. <i>lactis</i> N8	I	Valio Ltd, Graeffe et al., 1991
<i>Lactococcus lactis</i> ssp. <i>lactis</i> ATCC11454	I, II	ATCC, Schleifer et al., 1985
<i>Lactococcus lactis</i> ssp. <i>lactis</i> NZ9800	I	NIZO, Kuipers et al., 1993
<i>Lactococcus lactis</i> ssp. <i>lactis</i> LAC240	I	Reunanen and Saris, 2003
<i>Micrococcus luteus</i> A1 NCIMB8166	I, IV	ATCC, Hoff et al., 1947
<i>Weissella confusa</i> LAB10	IV	This work

Table 4. Plasmids used in this study.

Plasmid	Relevant properties	Studied in Paper nr	Plasmid origin and reference
pLEB579	Erm ^R 2.9 kb	III	This work
pLEB580	Erm ^R 4.2 kb	III	Takala and Saris, 2002
pLEB590	Nisin 3.1 kb	III	Takala and Saris, 2002
pLEB599	Erm ^R 7.5 kb	III	Reunanen and Saris, 2003
pNZ9111	Erm ^R 15.4 kb	III	NIZO, van der Meer et al., 1993

Erm^R = Plasmid encodes for erythromycin resistancy

3.2. Methods

Table 5. Methods employed in this study.

Method	Description	Found in Paper nr
<u>Strain isolation</u>		
<i>L. lactis</i> strains	M17G growth medium (Oxoid, Unipath Ltd, Basingstoke, England) containing 0.5% (w/v) glucose	I, II
Non-starter bacteria	Sugar-free peptone agar (FIL-IDF Standard method. Anonymous, 1971)	II,
Molds and yeasts	Yeast Glucose Chloramphenicol agar (YGC, Merck, Darmstadt, Germany)	II,
Coliforms	Violet Red Bile agar (VRB, Biokar Diagnostics, Lyon, France)	II,
<i>Lactobacillus</i> strains	Modified Lactobacillus Selective Medium, mLBS (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) without acetic acid.	III, IV
<i>Bacillus licheniformis</i>	Trypticase Soy agar, TSA (Becton Dickinson)	
<u>Identification</u>		
Partial 16S rRNA gene sequencing	Edwards et al., 1989 Institute of Biotechnology, University of Helsinki, Finland	I, III, IV
Alignment and identification data base	NCBI Blast Library (www.ncbi.nlm.nih.gov)	I, III, IV
Structural nisin gene analysis	Graeffe et al., 1991	I
Riboprinting analysis	Pirttijärvi et al., 1999 RiboPrinter TM Microbial Characterization System, Qualicon, DuPont, Wilmington, DE, USA RiboPrinter TM System Data Analysis Program, 2000 Bionumerics, Applied Maths, BVBA, 9830 Sint-Martens-Latem, Belgium	I
GFP-based nisin bioassay	Reunanen and Saris, 2003 Fluoroskan Ascent 374 scanning fluorometer computer-linked with Ascent version 1.2 software (Labsystems, Helsinki, Finland).	I
Metabolic characterization	Biolog manual, Biolog Inc., Hayward, CA, USA Microlog TM System, Biolog Inc., Hayward, CA, USA	I
Agar diffusion test	Tramer and Fowler, 1964	I, IV
Whole cell fatty acid analysis	MIDI Inc., Newark, DE, USA	III
Growth characterization		III, IV
<u>Exploitation</u>		
Sensory evaluation	Pliner and Hobden, 1992, Tuorila et al., 2001	II
Electrotransformation	Sambrook et al., 1989b	III

3.2.1. Sample origin and specimen collection

Samples of human milk ($n = 20$) were collected during the early lactation period (within 80 days of birth). The donors were healthy first-time deliverers and mothers with several children from southern Finland. Two milk samples were received within a month from one donor. The donors were requested to wipe skin area with an antiseptic and to collect milk by spraying it into sterile 50 ml test tubes (Cellstar, Greiner-Bio One GmbH, Frickenhausen, Germany) avoiding skin contact. The fresh milk was screened for LAB within hours of collection. (Paper I).

Seven dog breeds (German Shephard, Golden Retriever, Jack Russell Terrier, Dachshund, German Shorthaired Pointer, Border Collie and a mixed breed) were selected on the basis of registration statistics in the USA (Anonymous, 2002a) and in Scandinavia (Anonymous 2002b, Anonymous, 2002c) in conjunction with the Fédération Cynologique Internationale (FCI) breed nomenclature group (Anonymous, 2002d). Faecal samples from three healthy individuals from each breed were collected fresh in southern Finland in sterile 50 ml tubes (Cellstar, Germany). Information was compiled on the breed, year of birth, health condition, medication and diet (commercial dogfood or homemade food). Owners were invited to relate the diet and health of their pet. (Paper III).

Samples of chicken crop and small intestine of the breed male Ross 208 were obtained from Danisco Innovation, Kantvik, Finland. Bacteria were streaked on LBS to pure cultures and stored at 4°C. (Paper IV).

3.2.2. Growth and nisin production of *Lactococcus lactis* subsp. *lactis* LL3 in human milk and in infant formula

L. lactis strain LL3 was examined for its ability to metabolize lactose, an ingredient of milk, on carbohydrate-free M17 agar (Oxoid) supplemented with 50 µg/ml of bromocresol purple as pH indicator (Merck, Germany) and 2% (wt/vol) lactose, incubated overnight at 30°C. To observe the capacity of *L. lactis* strain LL3 to grow in infant formula (Tutteli, Valio Ltd, Finland) and in human milk, these were seeded with 1.5×10^7 cfu/ml and incubated aerobically without shaking for overnight at 30°C. The early lactational breastmilk stored at -20°C was thawed and then heated for 10 minutes at 70°C in a water bath to inactivate the microbiota. The strain LL3 was grown overnight at 30°C in M17G broth (Oxoid) containing 0.5% (wt/vol) of lactose. Growth was recorded in four replicate assays. (Paper I).

To test whether or not the antibacterial activity of the *L. lactis* strains identified in this study were nisin, a GFP-based nisin bioassay (Reunanen and Saris, 2003) was employed. In this bioassay, nisin was identified and quantified with fluorescence correlated with the nisin concentration in the samples. For this, the strains were grown in M17G broth containing 5 µg/ml of erythromycin (Erm), 0.1% Tween80 and LAC240 as an indicator strain for overnight at 30°C. Fluorescence (excitation 485 nm, emission 538 nm) was detected in terms of relative fluorescence units (RFU) with a Fluoroskan Ascent 374 scanning fluorometer (Labsystems, Helsinki, Finland), computer-linked with Ascent version 1.2 software (Labsystems). Viable count for all strains was measured on M17G plates (48 h, 30°C) before quantifying nisin production.

3.2.3. Fermenting soymilk with *L. lactis* LL3 and *L. lactis* ATCC11545

L. lactis strains LL3 and ATCC11545 were grown overnight at 30°C in M17G (Oxoid,) containing 0.5% (w/v) of glucose and then subcultured twice in UHT-treated unsweetened soymilk (Tofuline, Carlshamn Mejeri, Sweden) at an inoculation ratio of 1:40. *L. lactis* LL3 and *L. lactis* ATCC11545 (2.25×10^8 cfu/ml) were separately added with strawberry purée (1:100) into sweetened (3.5% glucose) and unsweetened (1.5% glucose) soymilk and left to ferment aerobically (stationary) overnight at 30°C. After adding the *L. lactis* inoculum and before fermenting, the pH of the mixture was 6.5. The strawberry purée consisted of fresh strawberries cooked with sucrose (30% w/v), jarred aseptically and stored at 6°C. The fermented products were cooled to 6°C and shelved at 6°C for three weeks. A survey was conducted to compare the consumer palatability and acceptability of the fermented soymilk (sweetened, Tofuline) products made with *L. lactis* strains LL3 and ATCC11545 and strawberry purée. Both products were refrigerated at 6°C overnight before serving. (Paper II).

3.2.4. Acid and intestinal content tolerance of lactic acid bacteria isolated from canine faeces

Tolerance to acidity at pH values 2, 4, and 7 was tested using survival at pH 5.7 (normal pH of mLBS broth) as reference. The acidity of mLBS was adjusted using 37% hydrochloric acid (Merck). Strains were cultivated in the presence of air at 30°C for 0, 2, 4, 8, 24 hours. Cross-inhibition was tested by cross-streaking each strain over each other on a mLBS plate and then grown overnight aerobically at 30°C. (Paper III).

3.2.5. Growth characterization of *Lactobacillus crispatus* strains

Resistance to erythromycin (Sigma Chemical Co, St. Louis, MO, USA) and to nisin (Sigma Chemical Co, St. Louis, MO, USA) as well as the sensitivity to glycine (Merck, Darmstadt, Germany) and growth under aerobic conditions in MRS broth (Becton Dickinson Microbiology System, Cockeysville, MD, USA) were measured as an increase of OD₆₀₀ (Lambda Bio UV/VIS spectrophotometer, PerkinElmer Inc, Boston, MA, USA). (Paper IV).

3.2.6. Electrotransformation of poultry originated *L. crispatus*

Transformation of genetic material by electroporation has been successfully employed with a number of bacterial species (Argnani et al., 1996; Holo and Nes, 1989; Serror et al., 2002). Electrotransformation is based on exposing log phase bacterial cells to electric pulse in order to porate the cell membrane. Since Gram-positive bacteria possess a thick cell membrane, glycine is employed in growth medium to weaken the membrane allowing the porous membrane genetic material, such as plasmids, to enter the cell. Bacteria are cultivated in recovering broth for several hours before plating on a selective medium (Sambrook et al., 1989b).

To initiate electroporation, an overnight culture of *L. crispatus* was diluted to 1% in fresh MRS broth and then grown again for eight hours at 37°C. Subculturing was repeated in MRS broth supplemented with 0.8% (w/v) glycine and harvested at OD₆₀₀ of 0.3-0.4 by centrifugation at 9000 × g (10 min, 4°C). The cells were washed twice in ice-cold electroporation buffer (0.5 M sucrose, 7 mM KPO₄, 1 mM MgCl₂, pH 7.4), resuspended in electroporation buffer to 1/100 of the original culture volume and stored on ice for a maximum of one hour. Plasmids were transformed into the *L. crispatus* strains as follows:

50 μ l of cell suspension and 500 ng of plasmid DNA were pipeted into 2 mm interelectrode-gap cuvettes (BTX CuvettesTM, San Diego, USA) and a 1.5 kV pulse (200 Ω , 25 μ F) was then applied to the cells (Gene PulserTM and Pulse Controller, Bio-Rad Laboratory, Richmond, USA). Cells were then incubated in 2 ml of MRS broth amended with 2 mM CaCl₂ and 20 mM MgCl₂ for three hours at 37°C. The cells containing plasmids were picked from MRS agar with 5 μ g of erythromycin /ml and cells with nisin selection were picked from MRS agar with 100 IU nisin /ml after three days of growth at 37°C. Negative controls without the plasmid DNA were similarly cultivated. Transformed colonies taken from the plates were placed in MRS broth with erythromycin for plasmid isolation. For analysis of the *L. crispatus* transformants, plasmids were isolated and retransformed into CaCl₂-treated *E. coli* TG1 cells due to the better plasmid quality when harvested from *E. coli*. The electrotransformed plasmids were analyzed on 1% agarose gel (BioCell Products Oy, Helsinki, Finland). (Paper IV).

4. Results and discussion

4.1. Isolation of lactic acid bacteria from human, canine and poultry sources

The human GI tract has been estimated to contain several hundred species of cultivatable bacteria (Moore and Holderman, 1974; Goldin, 1990), of which lactobacilli are numerically a minority (Tannock, 1990). Lactobacilli are thought to represent 0 - 2.4% of the human microbiota (Brown, 1977; Hove et al., 1999). Sghir et al. (2000) reported the amount of *Lactobacillus* sp. in human faeces is less than 2%. Molecular analysis demonstrates that individual humans differ in their microbiota (Favier et al., 2002; Kimura et al., 1997; McCartney et al., 1996). The faecal microbiota of monozygotic twin siblings were reported to be more similar than those of dizygotic twins (van de Merwe et al., 1983). In the view of the large inter-individual differences in the microbiota of human (Kimura et al., 1997), a single probiotic strain may not be equally effective in all individuals (Simmering and Blaut, 2001).

We isolated lactococci from human milk. In order to find LAB with antagonistic properties, we used on LB agar overlaid with *M. luteus* A1 NMIB86166 (Tramer and Fowler, 1964). Bacterial strains were selected from 20 samples of human milk based on large inhibition zones on agar diffusion plates. Seven samples of milk from six different mothers yielded strains with strong antibacterial activity *M. luteus* A1 NMIB86166. The bacterial count of this milk varied between 2×10^2 and 8.7×10^4 cfu/ml (average 1.4×10^3 cfu/ml). Of these, 30% displayed consistent antagonistic activity against *M. luteus* and tested positive when assayed for nisin using the specific gfp bioassay (Reunanen and Saris, 2004). Previously only few bacteriocin-producing LAB species have been isolated from human and animal microbiota (Robredo and Torres, 2000; West et al., 1979). Only a single nisin-producing *L. lactis* strain has appeared in human milk, (Heikkilä and Saris, 2003). The reason for higher counts of nisin-producing *L. lactis* in this study possibly derive from the plating technique, the plates used in previous studies being less favorable for isolating nisin-producing *L. lactis* strains.

Here, the lactobacilli from dog faeces were isolated on modified Lactobacilli Selective agar (mLBS) without acetic acid, a medium chosen for its superior selective properties (Benno et al., 1992; Gilliland et al., 1975). Plating faeces samples on LBS amended with acetic acid yielded no colonies, while plating on LBS without acetic acid did. Other selective media have been used, resulting in low amounts of LAB (Dahlinger et al., 1997). Also non-LAB species, such as *Pediococci* sp. and *Weissella* sp, survived on the medium with no acetic acid in our study. Of the 21 investigated samples of canine faeces, 14 (67%) contained culturable LAB, the amounts averaging $5.8 \times 10^5 \pm 2.1 \times 10^5$ cfu/g of wet faecal matter. The faeces of all tested dog breeds contained LAB with the exception of the Dachshund. An explanation for the lowered acid tolerance in the growth medium may be the shorter passage time in the canine intestine (Wingfield and Twedt, 1986) when compared to the human (Tortora and Grabowski, 1996).

L. crispatus strains from chicken crop and intestine were isolated on LBS agar (Apajalahti et al., 2001). Nine isolates were selected for study by electrotransformation with the type strain *L. crispatus* ATCC33820. A variety of *Lactobacilli* sp. has previously been isolated from chicken (Fuller and Brooker, 1974; Gilliland et al., 1975; Jin et al., 1996), *L. crispatus* being one of the identified strains (Edelman et al., 2002; Todoriki et al., 2001; van der Wielen et al., 2002).

We found LAB in human milk and among the intestinal microbiota of chicken and dog. Mammalian neonates receive their microbiota primarily in labor and later from the environment (Edwards and Parrett, 2002; Fuller, 1989; Fuller and Gibson, 1998; Metchnikoff, 1908), whereas LAB enter the GI tract of poultry soon after hatching and adhere to the crop epithelium throughout the life of the bird (Fuller, 1973). Chicks receive bacteria from the eggshell and the environment (Fuller, 1973) but are also fed bacterial preparation from adult intestinal microbiota to protect against pathogens, such as Salmonella (Nurmi and Rantala, 1973). LAB and bifidobacteria dominate the microbiota of the mammalian neonate, especially when breast-fed (Edwards and Parrett, 2002; Lönnerdal, 2000), with a health-promoting effect on the child (Arici et al., 2004; Boris et al., 1998; Edwards and Parrett, 2002). A study comparing the properties of LAB originating in fermented food with those in the GI tract showed that strains are able to attach *in vitro* to human enterocyte-like epithelial cells (Caco-2 cell line) and survive low pH and the presence of bile (Haller et al., 2001). The bacterial biota of the GI tract varies during first five months of an infant's life (Heilig et al., 2002) while in the adult the genera present is stable (Heilig et al., 2002), changing only with age (Savage, 1977). The strain composition varies between individuals (McCartney et al., 1996) as the results of Papers I and IV demonstrate. Microbiota shed from the bacteria-colonized intestinal epithelium can be detected throughout the GI tract (Tannock, 1990) and in faeces (Savage, 1977), indicating that LAB isolated from canine faeces have survived the GI tract of the host dog (Paper IV).

4.2. Identification of isolated strains of lactic acid bacteria

4.2.1. Genetic characterization of *L. lactis* and *Lactobacillus* sp.

Molecular methods are important for bacterial identification (Drancourt et al., 2000; Greetham et al., 2002; Heilig et al., 2002; Sghir et al., 2000) and possibly more accurate for LAB than are conventional phenotypic methods. In this study, a 600-900 bp segment of the 16S rRNA gene of the LAB isolates was sequenced and the sequence compared to strains in the National Center for Biotechnology Information (NCBI) Blast Library as well as to those of the respective type strains and to each other. Sequencing results revealed that six out of the 20 isolates obtained from the 20 investigated human milk samples were *Lactococcus lactis* (Table 6) with antimicrobial properties. Human milk contains bacteria (Pittard et al., 1991), mainly staphylococci and streptococci (Carrol et al., 1979; Eideman and Szilagyi, 1979; Heikkilä and Saris, 2003; West et al., 1979) and also LAB (Heikkilä and Saris, 2003; Martín et al., 2003). We found that nisin-producing strains of *L. lactis* were frequent in human milk (Paper I). The origins and functions of these *L. lactis* strains remain speculative, but the maternal skin has been proposed as a source (Carroll et al., 1979; Eideman and Szilagyi, 1979; Martín et al., 2003).

LAB were found in 67% of canine faeces samples (n = 171) (Paper IV). Based on the 16S rRNA gene sequence, the canine isolates were identified as *Lactobacillus rhamnosus* (n = 27, 16.2%), *Weissella confusa* (n = 27, 16.2%), *Pediococcus acidilactici* (n = 26, 15.6%), *Lactobacillus casei* (n = 21, 12.6%) and *Lactobacillus salivarius* (n = 18, 10.8%) as well as other species (n = 48, 28.6 %). The sequence similarities are shown in Table 6. Of the species isolated in this study, *L. salivarius*, *L. reuteri* and *L. murinus* were reported in canine faeces also by other authors (Fujisawa and Mitsuoka, 1996; Greetham et al., 2002). As far as is known, this thesis and Paper IV represent the first report of *L. rhamnosus*, *W. confusa*, *P. acidilactici*, *L. casei*, *L. mucosae* and *L. fermentum* in canine faeces.

All isolated LAB of chicken origin were identified as *Lactobacillus crispatus* (Paper III). Phylogenetic trees of *L. crispatus* strains based on partial 16S rRNA sequences (TreeTop – Phylogenetic Tree Prediction analyzed with pair distance (Blosum62, Anon, 2003)) and whole cell fatty acid analysis (Paper III) revealed a close relation between the *L. crispatus* strains from chicken crop and intestine (Table 6). *L. crispatus* has also been reported in poultry intestine in several other studies (Sarraf et al., 1985; van der Wielen et al., 2002).

Table 6. Species identification based on partial (600 to 900 bp) 16S rRNA gene sequence comparison. The obtained sequences were compared to those of the respective type strains in NCBI Blast Library (www.ncbi.nlm.nih.gov). Nucleotide sequence accession numbers for the partial 16S rRNA gene sequences (EMBL, Germany) and the origins of the strains are given.

Strain	Similarity %	Accession number	Strain origin
<i>Lactococcus lactis</i> LL3 (DSM14456, HAMB12371)	99.8	AJ419572	Human milk
<i>Lactococcus lactis</i> 310	99		Human milk
<i>Lactococcus lactis</i> 2410	99		Human milk
<i>Lactococcus lactis</i> 3A	99		Human milk
<i>Lactococcus lactis</i> 4B	99		Human milk
<i>Lactococcus lactis</i> 6A	99		Human milk
<i>Lactococcus lactis</i> 6B	99		Human milk
<i>Lactobacillus crispatus</i> 28mc	99	AJ421221	Chicken cecum
<i>Lactobacillus crispatus</i> 145mc	Nd		Chicken cecum
<i>Lactobacillus crispatus</i> 29mi	Nd		Chicken ileum
<i>Lactobacillus crispatus</i> 81mi	Nd		Chicken ileum
<i>Lactobacillus crispatus</i> 101mi	99	AJ421222	Chicken ileum
<i>Lactobacillus crispatus</i> 119mi	97.8	AJ421223	Chicken ileum
<i>Lactobacillus crispatus</i> 134mi	99	AJ421224	Chicken crop
<i>Lactobacillus crispatus</i> A33	100	AJ421225	Chicken crop
<i>Lactobacillus fermentum</i> LAB8 (DSM16347, HAMB12670)	99		Canine faeces
<i>Lactobacillus mucosae</i> LAB12 (DSM16355, HAMB12674)	97		Canine faeces
<i>Lactobacillus rhamnosus</i> LAB11 (DSM16349, HAMB12673)	98		Canine faeces
<i>Lactobacillus salivarius</i> LAB9 (DSM16357, HAMB12671)	97		Canine faeces
<i>Weissella confusa</i> LAB10 (DSM16348, HAMB12672)	97		Canine faeces

Nd = Not determined here. Strain sequence results obtained from Danisco-Cultor Ltd.

4.2.2. Antimicrobial activity of *L. lactis* isolated from human milk

Many different antimicrobial peptides, most commonly nisin, have been found in strains of *L. lactis* (Barefoot and Nettles, 1993; McAuliffe et al., 2001). We investigated isolates of *L. lactis* from human milk for their potential to produce nisin. The GFP based nisin bioassay (Reunanen and Saris, 2003) was employed for this purpose. In this bioassay, nisin is identified and quantified by fluorescence which is proportional to the concentration of nisin in the samples. We found that all human *L. lactis* isolates produced nisin (Paper I). The strain LL3 grew in heat-treated human milk and in UHT-treated infant formula at 30°C (Fig. 2), producing 3.75 µg nisin /ml and 5 µg nisin /ml respectively overnight. The quantities are sufficient to inhibit a number of pathogens (Thomas et al., 2000). Differences in strain growth in both types of milk after 24 h were not significant.

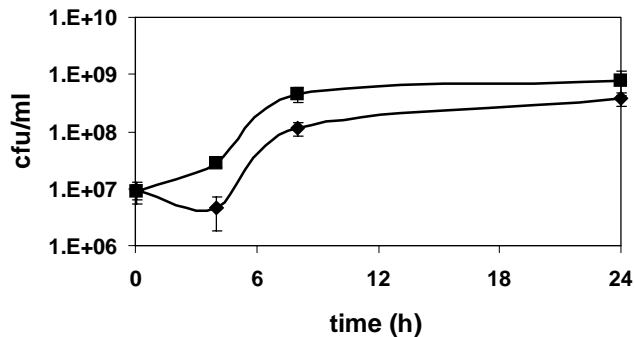


Figure 2. Growth of *Lactococcus lactis* LL3 of human origin in human milk (◆) and in infant formula (■) (Tutteli, Valio Ltd, Helsinki, Finland) at 30°C. Data points and standard errors represent averages of four replicates.

LAB strains of canine origin did not inhibit each other when cross-streaked on LBS agar. Also no antimicrobial effect was observed towards *M. luteus* A1 NCIMB86166 in the agar diffusion test (Paper IV). The five tested LAB strains demonstrated the capability to grow among each other, offering potential additive or synergistic probiotic impacts. A probiotic product including several strains provides a faster strain growth rate due to the superior performance of energy generation (Centeno et al., 2002) and offers a wider health benefit than would a single strain. Greetham et al. (2002) concluded that the lactobacilli isolated from canine faeces possibly constitute competitive exclusion to the benefit of the canine host.

4.2.3. Genetic characterization of the strains of *L. lactis* and *Lactobacillus* found in human milk

The seven nisin-producing strains of *L. lactis* isolated from human milk were compared by Riboprinting analysis to the *L. lactis* strains ATCC11454 and N8 producing nisin A or nisin Z, the two natural nisin variants existing in food. The ribopatterns obtained with *EcoRI* and *PvuII* enzymes of the isolates of human milk formed a cluster (Paper I, Fig. 1). The nisin producers of human origin differed from the strains producing nisin A and nisin Z isolated from cow milk. When the resulting fragment sizes from the two enzymes were combined and analyzed using the UPGMA algorithm, the human isolates clustered

separately from the respective type strains and strain N8. Sequencing to determine the nisin variant of the nisin structural gene of the *L. lactis* strain LL3 revealed it to be a nisin A producer (Paper I).

4.2.4. Strain characterization of the isolated lactic acid bacteria

Nisin-producing *L. lactis* strain LL3 was examined for its ability to metabolize lactose, an ingredient of milk and infant formulas. The use of other carbohydrates was screened to determine if strain LL3 grew in growth media other than human milk. (Paper I). When *L. lactis* LL3 was added as a protective culture to infant formulas (Gupta et al., 2000; Hurst and Hoover, 1993; Lee et al., 2003; Thomas et al., 2000; Zapico et al., 1998), it used lactose for growth. *L. lactis* LL3 fermented nine monosaccharides, six disaccharides and some amino sugars and glycosides, indicating that strain LL3 can ferment soymilk, a milk-like vegetable product. (Paper I). Soymilk is high in protein but also contains sucrose (Mital and Steinkraus, 1974), a disaccharide which *L. lactis* LL3 fermented (Paper I). Fermentation of soymilk is also known to mask its unappealing flavor (Mital and Steinkraus, 1974).

L. fermentum, *L. mucosae*, *L. rhamnosus*, *L. salivarius* and *W. confusa* of canine origin were found to be oxygen tolerant: the average culture density in mLBS broth reached an OD₆₀₀ of 1.4 (6.3×10^9 cfu/ml) within 12 hours of aerobic cultivation. *L. rhamnosus* achieved higher densities OD₆₀₀ ($1.6, 5.4 \times 10^9$ cfu/ml) in 12 hours at 30°C, whereas *L. rhamnosus* LAB11 incubated without oxygen rose to 4.1×10^9 cfu/ml in identical incubation conditions. (Paper IV). Stable and strong growth is desirable for the technological use of health-promoting bacteria (Charteris et al., 1998a; Knorr, 1998). The ambient concentration of oxygen did not effect the growth of the LAB strains isolated in this thesis, suggesting viability during the oxic phases of an industrial process. In order to survive until reaching the small intestine, probiotic bacteria must tolerate passage at low pH in the stomach. With this in mind, the five canine LAB strains were inoculated in mLBS to a density 10^6 to 10^8 cfu/ml and incubated for 24 hours aerobically at pH 2, 4, 5.7 and 7 at 30°C. All strains thrived in conditions of pH ≥ 4 . In addition, *L. salivarius* LAB8 and *W. confusa* LAB10 survived and grew to $>10^5$ cfu/ml within eight hours at pH 2, indicating that these acid-tolerant strains have a better chance of surviving the GI tract and thus show probiotic potential by being able to thrive in an industrial environment. *L. mucosae* LAB12 survived four hours at pH 2, while strains *L. fermentum* LAB8 and *L. rhamnosus* LAB11 did not survive longer than two hours when exposed to pH 2. (Paper IV). Acid tolerance was also reported for two *Lactobacillus brevis* strains taken from chicken ileum by Jin et al (1998), which tolerated pH 2 for three hours and survived for two hours even at pH 1.

The aerobic growth of *L. crispatus* strains was studied. Those tolerant to oxygen (28mc, 145mc, 29mi, 101mi, 119mi, 134 and A33) were investigated for sensitivity to erythromycin (1 µg of Erm /ml), nisin (10 IU of nisin /ml) and glycine (0.5 - 2%). These characteristics were required to monitor for tolerance to electrotransformation. Five *Lactobacillus* strains were selected for electrotransformation based on their resistance to 0.5% glycine and sensitivity to antimicrobials. (Paper III). Glycine in the growth medium has been reported to weaken the cell wall of Gram-positive bacteria in a dose-dependent manner (Holo and Nes, 1989; Thompson and Collins, 1996) and therefore aid electroporation.

4.3. Exploiting the novel isolates of lactic acid bacteria

In light of the documented biochemical, physiological and immunological influence of the GI microbiota on the host and its resistance to disease, the selection of health-promoting bacteria must rest on the documented impacts of selected probiotic strains on the microbial community harbored within the digestive tract (Tannock, 1998). These aspects are discussed below. Probiotics promote health in humans and animals through their presence in foodstuffs or pharmaceutical products (Bernet-Camard et al., 1997; Canducci et al., 2000; Gilliland et al., 1985; Gionchetti et al., 2000; Fooks et al., 1999; Heyman, 2000; Joosten et al., 1996, Kuisma et al., 2003; Nurmi and Rantala, 1973; Ryan et al., 1996; Ryan et al., 1998; Schaafsma et al., 1998).

4.3.1. Nisin-producing *L. lactis* LL3 of human origin improving the microbiological safety of infant formula

The protective property of 10^7 cfu/ml of *L. lactis* LL3 added to an infant formula was investigated *in vitro* against the toxin-producing *Bacillus licheniformis* 553/1 (Mikkola et al., 2000), as a consequence of a fatal food poisoning concerning an infant (Salkinoja-Salonen et al., 1999). We found that the strain LL3 inhibited the germination of *B. licheniformis* 553/1 spores and vegetative growth for 24 hours in an infant formula spiked with spores (10^2 cfu/ml) or vegetative cells (10^4 cfu/ml). *L. lactis* strains LL3 and NZ9800, a reference strain not producing nisin, were observed to multiply up to a hundred-fold aerobically in infant formula at 22°C and at 37°C in 24 h. In the presence of *L. lactis* NZ9800, *B. licheniformis* 553/1 displayed a lag phase of ≥ 6 hours before starting to grow in the formula. The number of vegetative cells of *B. licheniformis* 553/1 declined four log units in two hours, presumably due to the acid production of *L. lactis* NZ9800 and the consistency of the infant formula. When infant formula was inoculated with *L. lactis* LL3, *B. licheniformis* 553/1 declined to below the detection limit (<10 cfu/ml) in two hours and then remained at this level (Fig. 3). Nisin (1.25 µg/ml) was detected after two hours of incubation of the strain LL3 in formula. In eight hours at 37°C, the nisin concentration had reached 2.5 µg/ml. These data demonstrate that the inhibition of *B. licheniformis* 553/1 by *L. lactis* LL3 was due to nisin. Mansour et al. (1999) reported the inhibition of *B. licheniformis* in milk through the combination of nisin, monolaurin and pH 6. To increase shelf life of pasteurized dairy products significantly, 0.625 to 1.25 µg/ml of purified nisin was required (Delvis-Broughton et al., 1996), whereas 2.5 µg/ml was needed to inhibit the growth of *B. cereus* (Nissen et al., 2001). Our results indicate that *L. lactis* LL3 produced nisin concentrations sufficient to protect this dairy product against *B. licheniformis*, commonly present in infant formula as a contaminant (Rowan and Anderson, 1998).

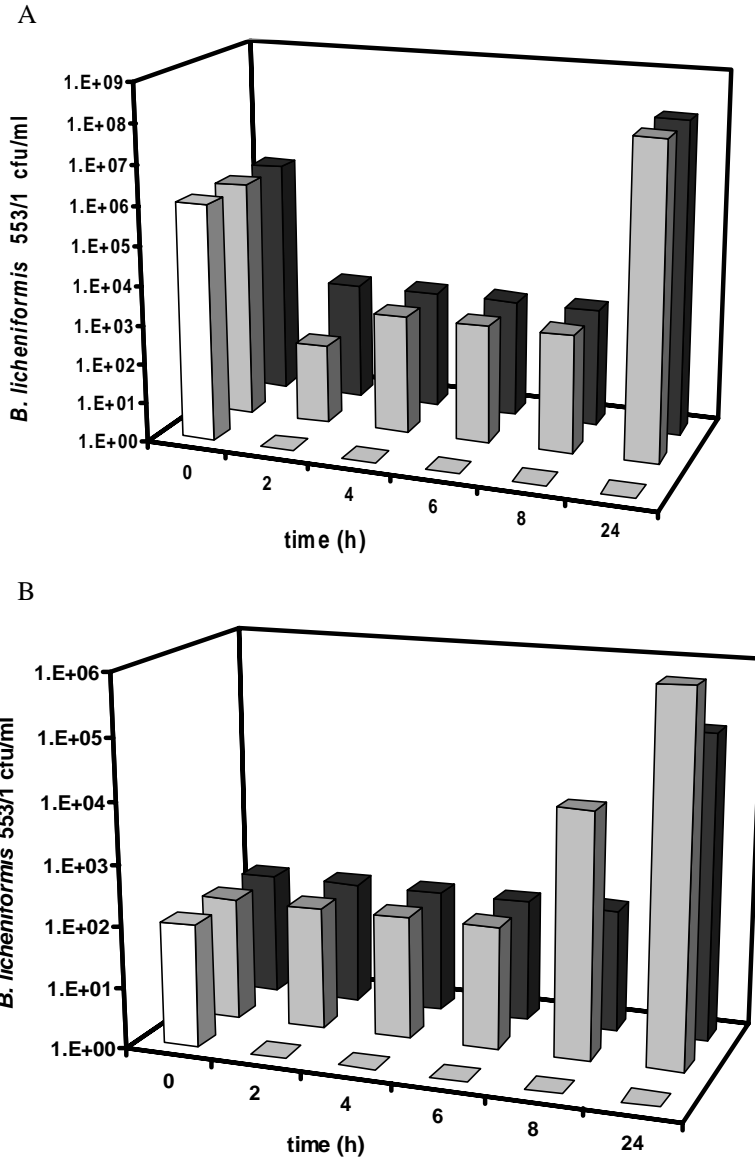


Figure 3. Growth of heat-stable toxin-producing strain of *Bacillus licheniformis* 553/1 in the presence of *Lactococcus lactis* LL3 in a commercial UHT-treated liquid infant formula (Tutteli, Valio Ltd, Helsinki, Finland). Inoculum was vegetative (A) *B. licheniformis* 553/1 or spores (B) of *B. licheniformis* 553/ grown at 37°C. A nisin non-producer strain of *L. lactis* NZ9800 served as a negative control. Black columns (no *L. lactis*); gray columns (*L. lactis* NZ9800); white columns (*L. lactis* LL3).

Many infants will consume nisin-producing *L. lactis* bacteria during breastfeeding (Paper I). Imbibing human milk containing nisin producers may positively affect infant health if nisin producers survive and if nisin is produced at levels inhibiting pathogens (Breukink and de Kruijff, 1999; Elliason and Tatini, 1999; Hurst and Hoover, 1993; Thomas et al., 2000; Zapico et al., 1998) in the digestive tract (Klijn et al., 1995; Drouault et al., 1999). Since *L. lactis* LL3 found in human milk is able to grow and produce nisin in infant formula and inhibits the growth of toxin-producing *B. licheniformis* 553/1 within two hours of inoculation (Paper I), this characteristic could be exploited by employing the nisin-producing *L. lactis* LL3 strain of human origin as an infant food protective culture against pathogens. Furthermore, nisin-producing *L. lactis* potentially protects mothers from mastitis and infants from infection by such pathogens as *Staphylococcus aureus* (Pittard et al., 1991). The effect of nisin producers on the microbiota of the human gastrointestinal tract remains to be clarified. Nisin producers might survive in the GI tract of human (Klijn et al., 1995) and of mouse (Drouault et al., 1999), but it is not known if nisin is produced in the intestine.

4.3.2. Nisin-producing *L. lactis* LL3 of human origin as a yoghurt starter

We used *L. lactis* strain LL3 isolated from human milk (Paper I) to ferment soymilk in parallel with *L. lactis* ATCC11545 originating from cow milk to determine if *L. lactis* LL3 can grow in soymilk and produce a palatable product for vegetarians or those allergic to milk. Both *L. lactis* strains survived at densities of $> 10^8$ cfu/ml during a three-week shelving of sweetened and non-sweetened UTH-treated soymilk at 6°C, despite a transient decline in *L. lactis* levels during the second week of storage. Adding glucose and/or strawberry purée to soymilk accelerated the growth of the *L. lactis* strains (Paper II). Chou and Hou (2000) and Rossi et al. (1999) also observed that soymilk with added carbohydrates supported the growth of several lactic acid bacteria cultured together. After ensuring adequate survival of the fermenting bacteria, the microbiological safety and the stability of the soymilk product was investigated (Paper II). No coliform bacteria, yeasts or molds were detected (<10 cfu/ml) except in one batch of sweetened soymilk with 1% strawberry purée where yeast and mold were detected at levels of 50 cfu/ml. The density of non-starter bacteria remained below 350 cfu/ml (\pm SD of 90 cfu/ml) after three weeks of storage, indicating good hygienic standard. The pH and sensory characteristics were stable throughout the three-week shelving period, with pH remaining at 4.4 on average and above 4.2 at all times. The density of *L. lactis* LL3 was stable in soymilk over the three-week shelf life at 6°C. A consumer survey was conducted at a food fair to compare the acceptability of the products fermented with *L. lactis* strain LL3 (human milk isolate) and ATCC11545 (originating from cow milk). In blind sampling, the two strains were rated equally attractive, whereas information on the origin of the LL3 product significantly enhanced its perceived pleasantness. (Paper II).

This thesis was the first to demonstrate the use of LAB isolated from human milk as a starter in food processing. The consumer survey demonstrated the positive attitude towards the origin of the starter, indicating that strains taken from human milk would be an acceptable source as a new starter or probiotic strain. These properties show potential for use as a health product for lactose-intolerant consumers, vegetarians and milk-allergy patients. Additionally, soymilk is free of cholesterol, gluten and lactose (Liu and Lin, 2000). The nutritive quality of soybean protein stands at the pinnacle of the food chain available in the plant world. The phytoestrogens in soy have been found to stimulate the clearance of cholesterol by up-regulating low-density lipoprotein (LDL) receptors, thereby

increasing LDL receptor activity (Tham et al., 1998) and so augmenting the health-benefit values of soy.

Our study showed that the monoculture of *L. lactis* LL3 was enough to stabilize the flavor of the product, leading to high ratings for “pleasantness” in taste and texture from a consumer tasting panel. A monoculture adapts better to industrial processes than multicomponent cultures, such as combinations of *Enterococcus faecium*, *Lactobacillus acidophilus*, *L. jugurti*, *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* added to soymilk (Rossi et al., 1999). Soymilk is a good medium for bacteria capable of fermenting sucrose and raffinose oligosaccharides, mainly raffinose and stachyose (Scalabrini et al., 1998). When raffinose oligosaccharides are passed into the large intestine, anaerobic microorganisms ferment them to cause flatulence (Guimarães et al., 2001). High-protein soymilk has also been shown to lower cholesterol when soybean protein completely replaces the proteins in cow milk (Sitorori et al., 1999). However, soymilk also contains a trypsin inhibitor (Bai et al., 1998) which depletes the dietary protein digestion in the intestine.

L. lactis LL3 grew in soymilk to produce a palatable and acceptable fermented product. The product was microbiologically and flavor stable over the three-week shelf life at 6°C. *L. lactis* LL3 can be used as a monoculture for fermenting soymilk into yoghurt with good sensory characteristics.

4.3.3. Lactic acid bacteria from healthy dogs

The LAB strains isolated from canine faeces (*L. fermentum* LAB8, *L. salivarius* LAB9, *W. confusa* LAB10, *L. rhamnosus* LAB11 and *L. mucosae* LAB12) grew under aerobic and anaerobic atmospheres and at pH 4 and above within 48 hours. LAB strains demonstrated the capacity to grow with each other, allowing for combined use to provide potential complementary or synergistic probiotic effects for dogs. (Paper IV). Since the probiotic addition to canine weaning food is thought to promote overall health over the dog’s lifespan particularly the development of its immune system (Benyacoub et al., 2003; Biourge et al., 1998). Dog foods containing LAB are available in pet stores and from veterinarians in Finland (Anon, 2004a; Anon, 2004b). The origins of these LAB have not been made public. In the absence of a safe and effective host-specific probiotic product for dogs, *E. faecium*, the sole pharmaceutical probiotic on the commercial market for veterinary use in Finland, was reported to increase the adhesion and colonization of *Campylobacter jejuni* on canine intestinal mucus (Rinkinen et al., 2003). LAB isolated from canine intestine adhere to immobilized intestinal mucus (Rinkinen et al., 2000) and inhibit the adhesion of certain bacterial pathogens (Rinkinen et al., 2003). Some enterococcal strains are opportunist pathogens with transferable antibiotic resistance and thus are not advised for use as probiotics in food (Sanders, 2003). The need for feeding studies is felt for evaluation of the potential probiotic impacts of the strains isolated and studied in Paper IV. The LAB strains discussed in Paper IV, present in healthy dogs, may possess the technical potential for serving as probiotics for dogs.

4.3.4. Electrotransformability of lactic acid bacteria of poultry origin

The suitability of *L. crispatus* strains (28mc, 145mc, 29mi, 101mi, 119mi, 134mi and A33) taken from chicken, as well as the type strain ATCC33820 was investigated for genetic engineering. Electrotransformation of pLEB579 yielded erythromycin-resistant colonies from four strains of *L. crispatus*, with a transformation frequency of 30

transformants per μg of DNA. Transformations were repeated from five to seven times, depending on the strain. Strains electrotreated without a plasmid and plated on MRS agar containing 5 $\mu\text{g}/\text{ml}$ of erythromycin did not yield erm resistant colonies. Electrotransformation (from one to seven attempts, depending on the strain) with the plasmids (pLEB580, pLEB590, pLEB599 and pNZ9111, their size varying from 3.1 to 15.4 kb) did not produce colonies, suggesting that the plasmid size had an impact on the transformation efficiency (Paper III). In most bacteria, the frequency of electroporation generally decreases with the increasing size of the transforming DNA (Gasson and Fitzgerald, 1994). A low frequency of transformation among wild-type lactobacilli is common (Aukrust and Blom, 1992). In conclusion, wild-type LAB are potentially electrotransformable when the DNA transformed is small enough.

Isolation of the plasmid (pLEB579) from the *L. crispatus*, erm^R transformants resulted in preparations in which the isolated DNA was extensively degraded (Paper III). For this reason, plasmid content analysis of the transformants was performed by transformation of the isolated plasmids into *E. coli* TG-1 from which the plasmids could be isolated without degradation. These plasmids were mostly of altered size and, as a rule, larger compared to plasmid pLEB579. Only a portion of the plasmids originating from the transformants of strains 101mi, 119mi and A33 were of the same size (2.9 kb) as pLEB579. These plasmids displayed the *Sau*3AI restriction pattern identical to pLEB579. The *L. crispatus* strains were observed to rearrange the plasmids efficiently (Fig. 3). To avoid these stability problems, expression constructs should aim at integration into the chromosome, a more stable location than multicopy plasmids. Our results revealed that genetic engineering of *L. crispatus* of poultry origin was possible (Fig. 3). In spite of the observed low frequency, regions of the plasmid, after further analysis, might provide the basis for the construction of efficient vectors that could be used to genetically modify strains of *L. crispatus*.

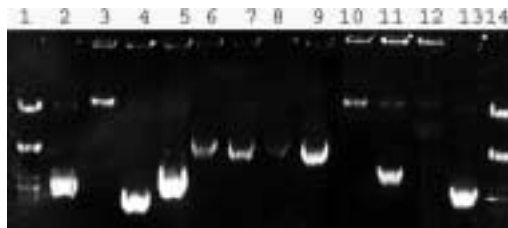


Figure 3. Rearrangements of selected plasmids electrotransformed into strains of *Lactobacillus crispatus*. A 2,9 kb plasmid (pLEB579) was electrotransformed into *L. crispatus* A33 (lanes 3-12), retransformed and isolated from *Escherichia coli* TG-1. Lanes 1, 14: λ Pst marker; lanes 2, 13: pLEB579.

L. crispatus strains 134mi and A33 have been reported to adhere to chicken epithelia (Edelman et al., 2002). Fuller (1973) demonstrated that lactic acid bacteria adhered to chicken intestinal epithelium after hatching, and remain and survived in the intestine throughout the life of the chicken (Todoriki et al., 2001), revealing probiotic potential. In this thesis, a small plasmid (pLEM579, 2.9 kb) was electrotransformed into four strains of *L. crispatus* isolated from chicken crop and intestine, respectively, using a modified transformation method. This method enables the genetic engineering of *L. crispatus* towards poultry strains.

5. Summary and conclusions

LAB are present in the microbiota of mammals and birds (Fuller, 1989). This thesis describes LAB isolated from human milk and canine faeces as well as from chicken crop and intestine. These bacterial strains were characterized and their suitability for exploitation charted.

Nisin-producing *Lactococcus lactis* strains (n = 6) were isolated from 20 samples of human milk. The nisin from a high (3.75 µg/ml) producer strain *L. lactis* LL3 was identified as nisin A. This strain ferments lactose contained in all milks and infant formulas. *L. lactis* LL3 displayed good sensory and technical characteristics as a yoghurt starter in infant formula. The strain also inhibited a toxin-producing strain of *Bacillus licheniformis* 553/1. The strain LL3 thus shows potential for improving the microbiological safety of infant formula. Nisin-producing bacteria were found to be naturally present in human milk, and is recognized to be a new protective mechanism for human milk. When *L. lactis* LL3 was used to ferment soymilk, it survived at levels of > 10⁷ cfu/ml for three weeks in the soymilk. A consumer survey revealed that the acceptability of the fermented product was similar to a product made with *L. lactis* ATCC11545 originally isolated from cow milk. In blind sampling, the two strains were rated equally attractive, whereas information on the origin of the LL3 product significantly enhanced its perceived pleasantness in panel ratings.

Lactic acid bacteria were isolated from the faecal microbiota of healthy dogs in the search to find strains for use as canine probiotics. LAB were found in canine faeces when plated on an acetic acid-free medium but not on a lactobacilli isolation medium containing acetic acid (LBS). LAB appeared in 67% of the canine faeces sampled from the seven most popular dog breeds in Finland, Sweden and the USA. The species *L. rhamnosus*, *W. confusa*, *P. acidilactici*, *L. casei*, *L. mucosae* and *L. fermentum* were isolated from canine faeces for the first time in this research. *Lactobacillus fermentum*, *Lactobacillus mucosae*, *Lactobacillus rhamnosus* and *Weissella confusa* strains were able to grow with and without oxygen and were acid tolerant. No decrease in cell viability of *W. confusa* was observed in a LBS medium at pH 2 within four hours.

Lactobacillus crispatus strains 28mc, 145mc, 29mi, 101mi, 119mi, 134mi and A33 from chicken crop and intestine origin and the type strain ATCC33820 were characterized for genetic engineering potential. All grew aerobically, were sensitive to erythromycin and nisin and were tolerant to glycine. Out of five investigated plasmids, a 2.9 kb plasmid (pLEB579) was successfully introduced into four wild-type *L. crispatus* strains (101mi, 119mi, 134mi and A33) of chicken origin with a transformation frequency of 30 transformants per µg of DNA. The frequency was low although sufficient to allow bioengineering of these strains.

In this thesis, *Lactococcus lactis* ssp. *lactis*, *Lactobacillus crispatus*, *Lactobacillus fermentum*, *Lactobacillus mucosae*, *Lactobacillus rhamnosus* and *Weissella confusa* were taken from the gastrointestinal tract and faeces of animals and from human milk, then characterized and investigated for their potential as probiotics for their hosts. In conclusion, LAB of human and animal origin may serve as bacteria potentially promoting host-specific health.

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