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PIRJE HÜTT

Functional properties, persistence, safety and efficacy of potential probiotic lactobacilli

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To my family

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I **Hütt P**, Shchepetova J, Lõivukene K, Kullisaar T, Mikelsaar M. Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. J Appl Microbiol 2006;100:1324–32.

II **Hütt P**, Andreson H, Kullisaar T, Vihalemm T, Unt E, Kals J, Kampus P, Zilmer M, Mikelsaar M. Effects of a synbiotic product on blood antioxidative activity in subjects colonized with *Helicobacter pylori*. Lett Appl Microbiol 2009;48:797–800.

III Kõll P, Mändar R, Smidt I, **Hütt P**, Truusalu K, Mikelsaar RH, Shchepetova J, Krogh-Andersen K, Marcotte H, Hammarström L, Mikelsaar M. Screening and evaluation of human intestinal lactobacilli for the development of novel gastrointestinal probiotics. Curr Microbiol. 2010;61:560–566.

IV **Hütt P**, Kõll P, Stsepetova J, Alvarez B, Mändar R, Krogh-Andersen K, Marcotte H, Hammarström L, Mikelsaar M. Safety and persistence of orally administered human *Lactobacillus* sp. strains in healthy adults. Beneficial Microbes 2011; 2:79–90.

V Songisepp E, Mikelsaar M, Rätsep M, Zilmer M, **Hütt P**, Utt M, et al., inventors. Isolated microorganism strain *Lactobacillus plantarum* TENSIA DSM 21380 as antimicrobial and antihypertensive probiotic, food product and composition comprising said microorganism and use of said microorganism for production of antihypertensive medicine and method for suppressing non-starter lactobacilli in food product (international patent application PCT EE2009/000005, priority date 13.05.2008, published 19.11.2009 WO2009138091; European patent EP2309870, Estonian patent EE05340, applicant and patent owner Bio-Competence Centre of Healthy Dairy Products).

VI Songisepp E, **Hütt P**, Rätsep M, Shkut E, Kõljalg S, Truusalu K, Stsepetova J, Smidt I, Kolk H, Zagura M, Mikelsaar M. Safety of a probiotic cheese comprising *L. plantarum* TENSIA according variety of health indices in different age groups. Journal of Dairy Science. 2012 (submitted).

Pirje Hütt has contributed to the following original publications:

Paper I: Planning, laboratory work, data analysis and writing of the paper.

Paper II: Study design, study performance, data analysis and writing of the paper.

Paper III: Laboratory work and participation in the writing of the paper.

Paper IV: Study design, study performance, laboratory work, data analysis and writing of the paper.

Paper V: Design of clinical studies, study performance, data analysis.

Paper VI: Study design, study performance, data analysis and participation in the writing of the paper.

ABBREVIATIONS

AAD	Antibiotic-associated diarrhea
AFLP-PCR	Amplified fragment length polymorphism polymerase chain reaction
ALAT	Alanine aminotransaminase
AP-PCR	Arbitrarily primed polymerase chain reaction
ASAT	Aspartate aminotransaminase
ATCC	The American Type Culture Collection
ATP	Adenine triphosphate
BabA	Blood group antigen binding adhesin
BDC–LDL	Baseline diene conjugates of low-density lipoprotein
BMI	Body mass index
CagA	Cytotoxin associated protein A
CFU/g	Colony forming units per gram
CLSI	Clinical and Laboratory Standards Institute
DBPCT	Double-blind, placebo-controlled trial
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
EC	European Commission
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	US Food and Drug Administration
FF	Functional foods
FHEL	Facultatively heterofermentative lactobacilli
FOS	Fructo-oligosaccharides
FP	Framework Program
FUFOSE	Functional Food Science in Europe
F6PPK	Fructose-6-P phosphoketolase
GI	Gastrointestinal
GLP-1	Glucagon-like peptide 1
GOS	Transgalacto-oligosaccharides
GRAS	Generally recognized as safe
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HIV	Human immunodeficiency virus
HDL	High-density lipoprotein
HpSA	*Helicobacter pylori* stool antigen test
hs-CRP	High sensitive C-reactive protein
IBD	Inflammatory bowel disease
ILSI	The International Life Sciences Institute
ITS-PCR	Internal Transcribed Spacer PCR
kb	Kilo base pairs

LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
MIC	Minimum inhibitory concentration
MRS medium	de Man-Rogosa-Sharpe medium
NADH oxidase	Reduced nicotinamide dinucleotide oxidase
NADPH oxidase	Reduced nicotinamide dinucleotide phosphate oxidases
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	The National Collection of Type Cultures
NO	Nitric oxide
NORIP	Nordic Reference Interval Project
NOS	NO synthase
OECD	The Organisation for Economic Co-operation and Development
OLT	Open-label trial
OHEL	Obligately heterofermentative lactobacilli
OHOL	Obligately homofermentative lactobacilli
oxLDL	Oxidized low-density lipoprotein LDL
PCR	Polymerase chain reaction
PCR-DGGE	PCR-denaturing gradient gel electrophoresis
PFGE	Pulsed-field gel electrophoresis
PYY	Peptide YY
QPS	Qualified presumption of safety
RAPD	Randomly amplified polymorphic DNA analysis
RDBPCCOT	Randomised, double-blind, placebo-controlled crossover trial
ROS	Reactive oxygen species
Real-time PCR	Real-time polymerase chain reaction
rep-PCR	Repetitive DNA element PCR
RNS	Reactive nitrogen species
SCFA	Short-chain fatty acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel-electrophoresis
TAA	Total antioxidative activity
TAC	Total antioxidative capacity
TAS	Total antioxidative status
TG	Triglycerides
UBT	The urea breath test
VacA	Vacuolating toxin
WBC	White blood cells
WHO	World Health Organization

1. INTRODUCTION

The microbiota of the human gastrointestinal (GI) tract plays a pivotal role in human health. Different biologic functions, such as digestion of essential nutrients, maturation of intestinal epithelial cells, and impact on baseline physiologic parameters, including systemic effects on blood lipids, inhibition of harmful bacteria, and stimulation of the immune system, have been attributed to the microbiota through careful scientific evaluations over many decades (McFarland, 2000b; Eckburg *et al.*, 2005; Canny and McCormick, 2008; Sharma *et al.*, 2011; Greenblum *et al.*, 2012).

However, the declined birth rates and longer life expectancy in developed countries have led to increased prevalence of chronic disorders like cardiovascular disease and different metabolic disorders (WHO, 2003). Moreover, several respiratory and foodborne infections, and chronic diseases like urinary tract and *Helicobacter pylori* infections are still emerging (Foxman, 2003; Azevedo *et al.*, 2009; Vouloumanou *et al.*, 2009; Newell *et al.*, 2010). Although the prevalence of *H. pylori* infection has been declining in Estonia (Oona *et al.*, 2004), gastritis, peptic ulcer disease and its general consequences on health such as gastric malignancies still need attention. All this requires population based new preventive approaches, namely infection control and improved nutrition.

Functional food is the food that contains some health-promoting components beyond traditional nutrients. Examples of functional food include foods comprising specific dietary fibre, foods with added biologically active substances such as antioxidants or phytochemicals and probiotics (Stanton *et al.*, 2005; Siro *et al.*, 2008). Probiotics are defined as live microorganisms which, when administered in adequate numbers confer a health benefit on the host (FAO/WHO, 2001). In clinical trials consumption of probiotic food comprising different lactic acid bacteria has shown several scientifically established and/or clinically proved health effects as prevention of particular infections and non-infectious disorders (McFarland, 2000a; Salminen, 2001; Minocha, 2009; Floch *et al.*, 2011). However, studies focusing on expression of functional properties of a probiotic on healthy persons are lacking.

The Department of Microbiology of the University of Tartu has participated in national and several international projects, namely in the EU 5th, 6th and 7th Framework Programs, in cooperation with other European universities for assessment of the functional properties, safety and efficacy of some commercial and potential probiotics of lactic acid bacteria.

The present thesis specifies on evaluation of the functional properties (antagonistic and antioxidative activity) of seven commercial probiotics included in the EU 5th FP "Microfunction" with 5 European universities and enterprises. Three strains and a fructo-oligosaccharides preparation were selected according to the results of *in vitro* experiment for efficacy testing of a synbiotic product. The aim was to assess the impact of the synbiotic after consumption of capsules containing commercial probiotics with prebiotic raftilose P95 on health indices,

biochemical markers and faecal microbiota through research conducted by different university partners. In the present study the efficacy was assessed by measuring blood oxidative stress related indices in healthy volunteers.

Next, screening of the putative probiotics which have been collected from various studies and deposited in the Human Microbiota Biobank (acronym: HUMB, registration number: 977) was performed. Colonizing potential *in vitro* was assessed. To determine the safety parameters of the selected *Lactobacillus* strains, *in vitro* tests and an animal model was applied.

The survival and persistence of these strains in healthy volunteers were assessed in clinical trials. In a human intervention trial, the safe consumption of the high doses of the lactobacilli was assessed according to a self-reported questionnaire and clinical parameters in cooperation with scientists of the Karolinska Institute, Sweden in the course of EU 6th FP "Biodefence".

The author of the current PhD thesis has also participated in the charactertization and safety testing of the probiotic strain *Lactobacillus plantarum* TENSIA (DSM 21380). This strain has been patented in Estonia (Estonian Patent No 05340) and the international patent application (PCT/EE2009/000005) has been filed. This probiotic is characterized by antimicrobial and antihypertensive effect and the author of the thesis has evaluated the functional properties of the strain and its safety in healthy volunteers. The collection and evaluation of the clinical data were performed at the Department of Microbiology and at the Department of Biochemistry of the University of Tartu and at the Bio-Competence Centre of Healthy Dairy Products LLC, Estonia.

2. REVIEW OF THE LITERATURE

2.1. Beneficial intestinal bacteria

The intestine harbors an ecosystem composed of the intestinal mucosa, the digestive secretions and the commensal microbiota. The normal gut ecosystem can efficiently block intrusion of many pathogenic bacteria. This has been termed 'microbial interference' or 'colonization resistance' (van der Waaij *et al.*, 1971).

Lactobacillus sp. and *Bifidobacterium* sp. are microorganisms that form part of the human microbiota, having an important role in the first line of defence against opportunistic and invasive pathogens (Stecher and Hardt, 2008).

Moreover, the diseases and disorders such as inflammatory bowel disease, irritable bowel syndrome and obesity are associated with human gut microbiota where aberrations could be improved by consuming probiotic lactobacilli and bifidobacteria (Fujimura *et al.*, 2010). The underlying mechanisms depend on particular functional properties of different strains of the mentioned genera and species.

2.1.1. *Lactobacillus* spp.

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria, many of them having received a generally recognized as safe (GRAS) or qualified presumption of safety (QPS) –status. These bacteria are widely found in nature, including the GI and urogenital tracts of humans and animals, and are present in many fermented foods like salted gherkins, marinated olives, capers and salami, and different milk based products such as cheese and yoghurt (Tannock, 2004).

LAB are gram-positive, acid-tolerant and non-spore forming cocci and rods. They are a heterogeneous group of bacteria comprising about 20 genera within the phylum *Firmicutes*. From the practical point of view the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* have been considered as the principal LAB (Kandler and Weiss, 1986; Klein *et al.*, 1998; Holzapfel *et al.*, 2001; Axelsson, 2004).

The genus *Lactobacillus* is the largest LAB group comprising over 145 recognized species and 30 subspecies (Bernardeau *et al.*, 2008; Claesson *et al.*, 2008). These numbers are constantly being re-evaluated on the basis of modern methods of molecular biology and whole genome-based techniques (Makarova *et al.*, 2006; Felis and Dellaglio, 2007).

The classical way to distinguish between species of lactobacilli is based on the phenotypic properties of lactobacilli. According to carbohydrate fermentation patterns and growth at certain temperatures, the genus *Lactobacillus* is divided into homofermentative lactobacilli (OHOL), facultatively heterofermentative lactobacilli (FHEL), and obligately heterofermentative lactobacilli

(OHEL) subgroups (Kandler and Weiss, 1986; Klein *et al.*, 1998). The carbohydrate metabolism of lactobacilli usually allows taxonomic differentiation between species with the use of commercial kits (like API 50 CHL by bioMérieux).

A previous study has shown discrepancies in the identification of lactobacilli by API and Internal Transcribed Spacer PCR (ITS-PCR). The species of one-third of *Lactobacillus* strains were renamed according to the results of molecular typing techniques (ITS-PCR) (Annuk *et al.*, 2003).

Moreover, if one uses 16S phylogeny, then the *Lactobacillus* species can be divided into three groups: the *L. casei-Pediococcus* group, the *Leuconostoc* group and the *Lactobacillus acidophilus/delbrueckii* group (Collins *et al.*, 1991; Stiles and Holzapfel, 1997). Since then, based again on 16S rDNA sequences, it was proposed to divide the *Lactobacillus* species into five groups, namely *L. acidophilus*, *L. salivarius*, *L. reuteri*, *L. buchneri* and *L. plantarum* (Schleifer and Ludwig, 1995).

However, these classifications have generally been considered as unsatisfactory and the use of 16S rRNA genes as phylogenetic markers has been criticized (Claesson *et al.*, 2008). As complete genome sequences have become available, the high diversity of *Lactobacillus* has also been suggested to require the creation of new, subgeneric divisions (Canchaya *et al.*, 2006; Claesson *et al.*, 2008).

2.1.2. *Bifidobacterium* spp.

Bifidobacteria can be found all along the GI tract and the main species present in humans are *Bifidobacterium adolescentis*, *B. bifidum*, *B. infantis*, *B. breve* and *B. longum* (Crociani *et al.*, 1996; Marteau *et al.*, 2001; Nielsen *et al.*, 2003). Bifidobacteria are gram-positive, heterofermentative, non-motile, non-sporulating bacteria. They vary in size and exhibit rods of various shapes, often in 'V' or 'Y' patterns (Latin *bifidus*: cleft, divided). Bifidobacteria are often included in the group of LAB due to their metabolic capacities and the sharing of ecological niches (Klein *et al.*, 1998).

The genus *Bifidobacterium* is relatively small with ca 32 identified species to date, mainly of human or animal origin and has a low level of phylogenetic and genomic diversity (Ventura *et al.*, 2006). Bifidobacteria belong to the phylum *Actinobacteria* (Ventura *et al.*, 2007). Bifidobacteria are reported to possess a route for the metabolism of hexose, the fructose-6-P phosphoketolase (F6PPK) pathway (the so-called bifid shunt) (de Vries *et al.*, 1967). Lactic and acetic acids are produced as metabolic end products from hexose fermentation. F6PPK activity is one of the main phenotypic features used to identify bifidobacteria at the genus level. The second pathway involves the splitting of pyruvate by a phosphoroclastic enzyme to form formic acid and acetyl phosphate (Scardovi, 1986).

2.2. Gastrointestinal pathogens

GI pathogens infect the host in different ecological niches with particular environmental conditions of the GI tract. *Helicobacter pylori* resides under microaerobic conditions in the stomach. *Salmonella* sp. causes inflammation in the ileum and colon, while *Shigella* sp. clearly prefers the more anaerobic conditions on the colonic mucosa (Dupont, 2005; Pegues *et al.*, 2005). In addition, the colon has been considered the main reservoir of *E. coli* strains causing urinary tract infections (Franz and Horl, 1999).

2.2.1. Gastric pathogen *Helicobacter pylori*

Helicobacter pylori is a gram-negative, spiral-shaped, non-spore-forming microaerophilic bacterium that resides in harsh conditions of the gastric cavity and mucosa. *H. pylori* is associated with severe gastric pathologies, including chronic active gastritis, peptic ulcer, gastric adenocarcinoma, and type B low-grade mucosa-associated lymphoid tissue lymphoma (Dunn *et al.*, 1997; Kandulski *et al.*, 2008). *H. pylori* is classified as grade 1 carcinogen by the World Health Organization in 1994 (IARC, 1994). It has been suggested that *H. pylori* infection is also associated with several extragastric systemic diseases (cardiovascular and neurodegenerative diseases) and ocular diseases, and dermatological disorders (Izzotti *et al.*, 2009). DNA damage and failure of antioxidant defences is a common denominator of many among these pathological conditions. The clinical outcome of *H. pylori* infection is dependent on many variables, including *H. pylori* genotype, host health status, host genotype, and host exposure to environmental factors (Kandulski *et al.*, 2008). Infection with *H. pylori* invariably leads to a chronic inflammatory response (chronic active gastritis with lymphocytes infiltration), yet most infected patients remain asymptomatic (Dunn *et al.*, 1997).

Approximately 50% of the world's population is infected with *H. pylori* (Go, 2002). Various risk factors for infection include lower socioeconomic status, younger age, and geographic location (Bruce and Maaroos, 2008). The prevalence of *H. pylori* is decreasing in developed countries, while it is still high in developing countries (Hunt *et al.*, 2011). Infection is usually acquired in childhood and in the absence of antibiotic therapy persists for the life of the host (Everhart, 2000).

There are two types of diagnostic tests used to detect *H. pylori* infection: noninvasive and invasive. Noninvasive tests include the urea breath test (UBT), stool antigen tests and serology blood tests. Noninvasive tests detect the presence or absence of infection. Invasive tests include performance of upper gastrointestinal endoscopy with gastric biopsy. Invasive tests can determine both presence/absence of infection and the extent and severity of mucosal injury (Monteiro *et al.*, 2009).

Although several diagnostic tests are available for the detection of *H. pylori* infection, all of them have both advantages and disadvantages, and none can be considered as a single gold standard. A combination of endoscopic biopsy-based methods (such as rapid urease testing, histologic examination, culture, and PCR) usually provides the most reliable diagnosis (Maaroos *et al.*, 2004).

However, these methods are invasive, expensive, and not always applicable. Therefore, there is an increasing interest in non-invasive tests for *H. pylori* detection. The UBT is considered to be the most accurate non-invasive method to detect *H. pylori* infection. Stool antigen tests have been extensively evaluated in pre- and post-treatment settings both in adults and children (Dzierzanowska-Fangrat *et al.*, 2006). Monoclonal stool antigen test (*e.g.* HpSA) is an accurate noninvasive method both for the initial diagnosis of *H. pylori* infection and for the confirmation of its eradication after treatment. The monoclonal technique has higher sensitivity than the polyclonal technique, especially in the post-treatment setting (Gisbert *et al.*, 2006).

2.2.2. Other enteric pathogens

Salmonella sp., *Shigella* sp. and *Escherichia coli* are gram-negative, facultatively anaerobic, rod-shaped bacteria. Different *Salmonella* serovars are responsible for human diseases ranging from gastroenteritis to systemic infections (Falkow, 2006). *Shigella* species, particularly *S. sonnei* and *S. flexneri*, cause shigellosis in developed countries and globally (Deer and Lampel, 2010). Due to different virulence factors of *E. coli*, various infections may appear: gastroenteritis, urinary tract infections, meningitis, and wound infections (Kaper *et al.*, 2004).

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacillus. The symptoms of *C. difficile* infection can range from mild diarrhea to serious manifestations such as pseudomembranous colitis, toxic megacolon or perforation of the colon. *C. difficile* causes commonly nosocomial antibiotic-associated diarrhea (AAD) but it may also be related to community-acquired infection with no previous exposure to antibiotics in children, pregnant women and patients with inflammatory bowel disease (IBD). In recent years, a hypervirulent strain (*e.g.* NAP1/BI/027), with fluoroquinolone resistance has emerged. It may lead to severe GI infection and even to mortality (Ananthakrishnan, 2011).

2.3. Oxidative stress

Oxygen has a central role in the evolution of aerobic life on Earth. Aerobic organisms use molecular oxygen to generate chemical energy in the form of adenine triphosphate (ATP). However, oxidation may result in overproduction of toxic molecules, *e.g.* reactive oxygen species (Buonocore *et al.*, 2010).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS, *e.g.* nitric oxide, NO) are well recognised for playing a dual role as both beneficial and deleterious species. ROS and RNS are normally generated by tightly regulated enzymes, such as NO synthase (NOS) and NAD(P)H oxidase isoforms, respectively. The beneficial effects of ROS/RNS occur at low/moderate concentrations and involve a physiological role in cellular responses to noxia, as for example, in defence against infectious agents, in the function of a number of cellular signalling pathways, and in the induction of mitogenic response (Valko *et al.*, 2007). In contrast, overproduction of ROS (arising either from the mitochondrial electron transport chain or excessive stimulation of NAD(P)H) results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including membranes and molecules as lipids, proteins, DNA and carbohydrates.

Oxidative stress is defined as an imbalance between pro-oxidants (ROS, RNS) and antioxidants in favor of pro-oxidants (Sies and Cadenas, 1985; Halliwell, 2009). This imbalance may be due to an excess of pro-oxidant agents, a deficiency of antioxidant agents or both factors simultaneously.

An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of infections, cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive sleep apnea, and other diseases (Dröge, 2002).

The human body has evolved an integrated antioxidant defence system consisting of nonenzymatic antioxidants, such as reduced glutathione (GSH), vitamin E, C, Q_{10}, blood albumin, uric acid, bilirubin and enzymatic antioxidants (*e.g.* superoxide dismutase, glutathione peroxidase, catalase and heme oxygenase) (Gutteridge and Halliwell, 2000). An increase in antioxidants might be interpreted as the situation with "lower oxidative stress" (Bashan *et al.*, 2009).

Oxidative stress is also defined as 'a disruption of redox signalling and control' that emphasizes an impact of GSH and its redox ratio (GSH/GSSG) as good tools for the quantification of oxidative stress (Jones, 2006).

Determination of oxidative stress is mainly based on determination of oxidatively modified compounds (*e.g.* oxidized low-density lipoprotein (oxLDL), baseline diene conjugates of low-density lipoprotein (BDC-LDL), and antioxidative capacity of blood sera (*e.g.* total antioxidative activity (TAA) and total antioxidative status (TAS) (Ahotupa *et al.*, 1998; Ahotupa and Asankari, 1999; Kullisaar *et al.*, 2003; Songisepp *et al.*, 2005; Kullisaar *et al.*, 2011).

2.4. Functional food

Diet and nutrition are important factors in the promotion and maintenance of good health throughout the entire life-course. However, rapid changes in diets and lifestyles have a significant impact on the health and nutritional status of

populations. While the standards of living have improved, food availability has expanded and become more diversified. There have also been significant negative consequences in terms of inappropriate dietary patterns, decreased physical activities and increased tobacco use, and a corresponding increase in non-communicable diet-related chronic diseases (*e.g*. obesity, diabetes mellitus type 2, cardiovascular disease, hypertension and stroke, and some types of cancer) (WHO, 2003).

Functional food (FF) is a natural food, to which a component has been added/removed or a food in which the bioavailability of the components has been modified by technological or biotechnological means (Roberfroid, 2000a). FF includes conventional foods, modified foods (fortified, enriched, or enhanced), medical foods, and foods for special dietary use (Siro *et al.*, 2008; Hasler and Brown, 2009). FF can play an important role in the risk reduction of non-communicable diseases and can prolong remission in IBD (including Crohn's disease and ulcerative colitis) and alleviate allergic conditions by providing benefits beyond usual nutrition as well as in optimising health and general well-being (ILSI, 2009; Fujimura *et al.*, 2010).

The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) proposed a working definition of functional food: a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern: it is not a pill, a capsule or any form of dietary supplement (EC, 2010).

Essential attributes or characteristics of functional foods are the following: form and sensory characteristics (colour, texture, consistency and flavour, including appearance in conventional food). Second, contain nutrients and/or other substances that confer a physiological benefit over and above their basic nutritional properties. Third, possess functional benefits that can be scientifically proven and accepted by the relevant regulatory authority. Fourth, possess functional benefits that can be derived by consuming normal amounts of the foods. Fifth, contain an adequate amount of 'functional' nutrients and/or other substances that produce the claimed effect/in relation to the claimed effect and last, have been proven to be safe during long term usage by the intended target population, based on existing science. FF should not be intended for medical or therapeutic use (ILSI, 2009).

The most promising targets for FF are the GI functions and particularly control of nutrient bioavailability (Roberfroid, 2000b). However, FF may affect different other systems in the body: balanced colonic microbiota, control of transit time and mucosal motility, bowel habits; modulation of epithelial cell proliferation, balance of redox and antioxidant systems, metabolism of macronutrients, especially amino acids, carbohydrates and fatty acids.

2.4.1. Probiotics

Probiotics (the name is derived from the Greek 'for life') have been used for about 100 years. Today they are applied for treatment of a variety of infections of the intestinal tract or the vagina, where the use of antibiotics coincides with adverse shifts in microbial ecology. Moreover, these agents are sometimes being reconsidered as alternatives to antibiotics because of the rise in antibiotic-resistant strains of bacteria (D'Souza *et al.*, 2002). The term 'probiotic' was initially used in the 1950s and the definitions of probiotic have been refined as more experience has been gained, the most widely used being 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). Different definitions of the first generation of probiotics are listed in Table 1.

Table 1. Descriptions and definitions of probiotics suggested over time.

Published definition	Reference
Probiotics are opposite of antibiotics.	Vergin, 1954
Substances produced by microorganisms which promote the growth of other microorganisms.	Lilly and Stillwell, 1965
Organisms and substances that contribute to intestinal microbial balance.	Parker, 1974
A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.	Fuller, 1989
A viable mono- or mixed-culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora.	Havenaar and Huis in't Veld, 1992
A live microbial culture or a cultured dairy product which beneficially influences the health and nutrition of the host.	Salminen, 1996
Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition.	Schaafsma, 1996
A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as by improving nutritional and microbial balance in the intestinal tract.	Naidu *et al.*, 1999
A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host.	Schrezenmeir and de Vrese, 2001
Live microorganisms which, when administered in adequate amounts confer a health benefit on the host.	FAO/WHO, 2001
A probiotic product is a strain-specific preparation targeting different human metabolic functions to improve health by either supporting host physiologic activity or by reducing the risk of disease.	Mikelsaar *et al.*, 2011

Today, there is increasing interest in developing the second generation of probiotics, so called novel genetically engineered probiotics, or "designer probiotics" to combat pathogenic microorganisms (Ahmed, 2003; Celec *et al.*, 2005; Sleator and Hill, 2009) or to help alleviate inflammatory processes in the colon (IL-10 or trefoil factor secreting strains) (Steidler and Rottiers, 2006).

Probiotics provide beneficial effects on the host's health by affecting the intestinal microbiota. Their beneficial effects on human health, such as alleviation of lactose intolerance, immunomodulation, decrease in faecal carcinogenic enzymes and mutagenicity, hypocholesterolemic effect, and shortening of the duration of acute infectious diarrhea have been demonstrated in many studies (Salminen, 2001; Shah, 2007; Delcenserie *et al.*, 2008; Vasiljevic and Shah, 2008; Allen *et al.*, 2010).

LAB including lactobacilli and bifidobacteria are commonly used as probiotics, either as monostrain or multistrain (or multispecies) microorganisms (Timmerman *et al.*, 2004). Also other microbial species besides LAB like *Enterococcus faecalis, Escherichia coli, Propionibacterium* sp. and yeasts (*Saccharomyces boulardii*) have been accepted and used as probiotics (Prado *et al.*, 2008).

Probiotics can be administered as a component of functional food (yoghurt, cheese, milk) or as food additives (*e.g.* capsules, tablets). For the general population, outpatient and hospital patient probiotics have been applied. There is a need for more comparative clinical studies where the dosage, and number of different strains in combination could be evaluated for their survival, colonization potential and safety for the host.

2.4.2. Prebiotics

A prebiotics formula can be included in a wide range of foods, such as bakery, and dairy products and beverages. Prebiotics were originally defined as 'a non-digestible 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 that can improve the host health (Gibson and Roberfroid, 1995). Generally, prebiotics are specific oligosaccharides or more complex saccharides that are selectively metabolized for different compounds by some commensal groups of microorganisms, including species considered to be beneficial for the host.

The concept of prebiotics has been formalized by the establishment of three scientific criteria for a food ingredient to be considered as prebiotic (Gibson *et al.*, 2004): (i) resistance to gastric acidity and hydrolysis by mammalian enzymes and GI absorption; (ii) substrate of fermentation by intestinal microorganisms belonging to the human microbiota; and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing.

Prebiotics include nondigestible fructo-oligosaccharides (FOS) (*e.g.* inulin), oligofructose and transgalacto-oligosaccharides (GOS) (Candela *et al.*, 2010).

With an impact on the composition and metabolism of gut microbiota, prebiotics and their derived metabolites can exert different functional properties, such as prevention of pathogen adhesion and colonization; modulation of bowel habits; regulation of lipid and glucose metabolism (Laparra and Sanz, 2010).

It has been recently reported that oligofructose supplementation lowers hunger, promotes weight loss and improves glucoregulation in obese and healthy adults (Parnell and Reimer, 2009).

The mechanism by which the nutritional modulation of the gut microbiota by prebiotics impacts on appetite sensation is poorly understood, however, a fascinating hypothesis has been advanced (Cani *et al.*, 2009). Particularly, the increase in short-chain fatty acid (SCFA) production due to oligofructose fermentation by intestinal microorganisms may stimulate the biosynthesis of the satiety inducing hormones, such as glucagon-like peptide 1 (GLP-1), peptide YY (PYY) and ghrelin.

Earlier there have also been reported the positive effects of prebiotic consumption such as reduction of osteoporosis by improved mineral absorption; reduction of allergy risk through immune system modulation and reduction of colon cancer risk (Roberfroid, 2000b). Many of the above mentioned health claims still require further research.

2.4.3. Synbiotics

Prebiotics are the most frequent target substance for bifidobacteria and lactobacilli. The term synbiotic is used when referring to a product that contains a prebiotic and a probiotic in combination (Roberfroid, 1998). Synbiotics are mixtures that improve the survival and implantation of live microbial dietary supplements in the GI tract, either by stimulating growth or by metabolically activating health promoting bacteria (Kaur *et al.*, 2002). Saulnier and coworkers, studying various compositions of probiotic bacteria and prebiotics, have shown that certain synbiotics may have superior effects, compared with probiotics alone, in modulating the faecal microbiota (Saulnier *et al.*, 2008).

There are reports indicating the efficacy of a synbiotic supplementation in prevention of common winter diseases in children and improvement in the clinical appearance of active ulcerative colitis (Furrie *et al.*, 2005; Cazzola *et al.*, 2010). However, there is a lack of studies where the impact of different functional properties of probiotics and the metabolic pool of prebiotics on metabolic indices and oxidative stress related markers are properly investigated in clinical efficacy studies.

2.5. Regulation and guidelines for the evaluation of probiotics

2.5.1. EU regulation on nutrition and health claims

Regulation (EC) No 1924/2006 of the European Parliament and Council of 20 December 2006 on nutrition and health claims made on foods covers all foods, including food supplements and foodstuffs for particular nutritional uses, and concerns nutrition and health claims in advertisements, labelling and presentation of foods to consumers. Regulation 1924/2006 identifies two categories of claims on foods: nutrition claims and health claims. In the context of EU Regulation 1924/2006, health claims are claims that state, suggest or imply a relationship between a food or food category and health. Examples hereof are function claims, reduction of risk of disease claims, or claims referring to the growth and development of children. Nutrition claims are claims that state, suggest or imply that a food has particular beneficial nutritional properties due to the energy it provides or the nutrients it contains (EC, 2006; Verhagen *et al.*, 2010).

Health claims on (functional) foods must be scientifically substantiated. The European Food Safety Authority (EFSA) provides scientific advice to the European Commission for health claims (Verhagen *et al.*, 2010). The settled regulations also demand more scientific evidence based research regarding probiotics and prebiotics.

2.5.2. Guidelines for evaluation of probiotics

A joint working group of the FAO and the WHO developed guidelines to assess the efficiency and safety of probiotics in food. The FAO and WHO focused on guidelines and recommendations for the criteria and methodologies required to identify and define probiotics and to establish the minimum requirements needed to accurately substantiate health claims (Figure 1) (FAO/WHO, 2002).

2.5.2.1. Screening of putative probiotic strains

To screen intestinal lactobacilli strains for their advantageous properties for development of novel gastrointestinal probiotics involves a step-wise process in order to obtain a functional and safe product.

Though the genus *Lactobacillus* has a Generally Recognized As Safe (GRAS) and qualified presumption of safety (QPS) status, and a long history of safe use for food fermentation, several criteria must be taken into consideration to select and evaluate a putative probiotic *Lactobacillus* strain (FAO/WHO, 2002; Reid *et al.*, 2003; EFSA, 2011b). The properties of a putative probiotic must be thoroughly described *in vitro* as well as *in vivo* animal studies and in clinical trials.

Selection criteria for probiotics are an area of much debate and should be taken into account when defining appropriate strains. The following criteria have been suggested for use in probiotic strain selection (Figure 1) (Saarela *et al.*, 2000; FAO/WHO, 2002; Reid *et al.*, 2003; Vankerckhoven *et al.*, 2008):

1. General aspects (origin, identity)
2. Safety
3. Functional features
4. Technological aspects

The minimium requirements needed for probiotic status include the assessment of strain identity, *in vitro* tests to screen potential functional properties and colonizing potential of probiotics, assessment of safety above all, and *in vivo* studies for substantation of effects.

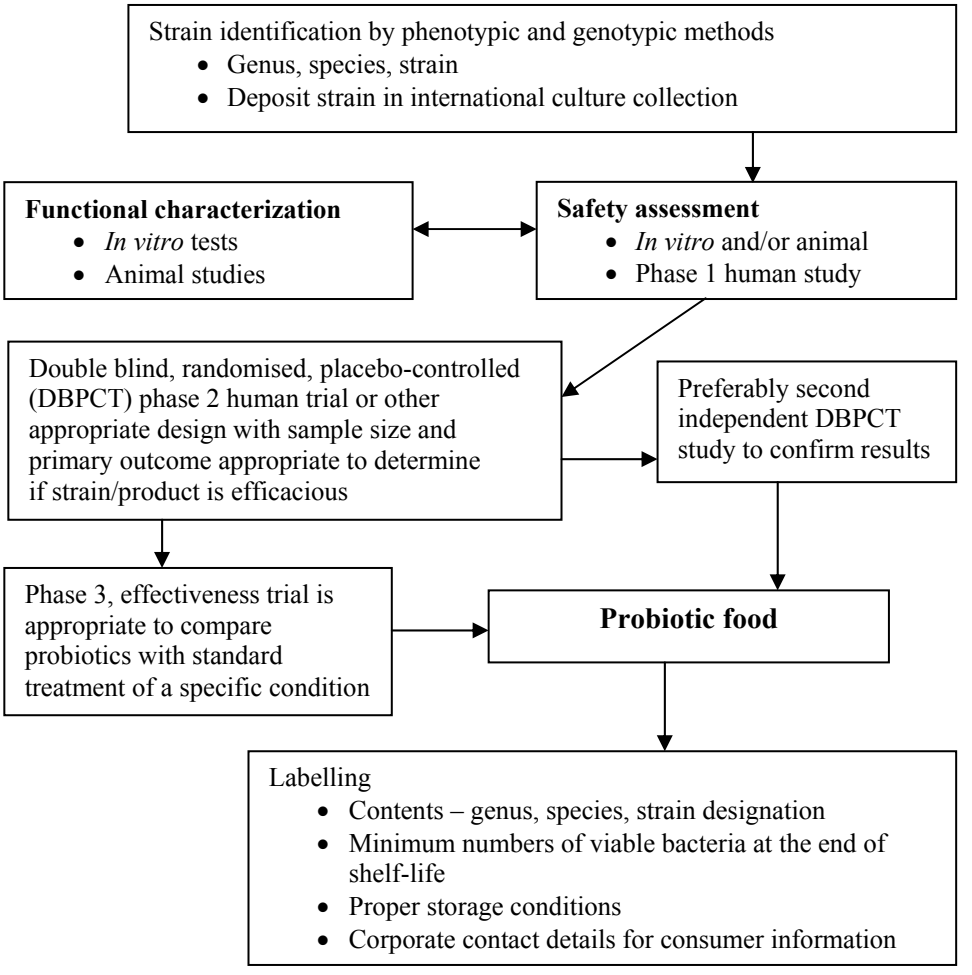


Figure 1. FAO and WHO (2002) guidelines for evaluation of probiotics for food use.

2.5.2.2. Origin, identification and typing of strains

A microbial strain retains its functionality in an environment similar to that from which it was originally isolated. Therefore, a probiotic strain aimed for human use should be preferably isolated from the healthy human GI tract (Saarela *et al.*, 2000).

Correct assessment of taxonomic identity should be scheduled in the screening process for new probiotic candidate strains (Saarela *et al.*, 2000; FAO/WHO, 2002; Vankerckhoven *et al.*, 2008). Correct identification of probiotic strains to the species level is essential for safety assessment as it allows a linkage to potentially relevant, species-related scientific and technological information, including data on growth conditions, metabolic characteristics and genomic information. In addition, strains must be identified to the strain level (FAO/WHO, 2002).

The ability to identify a specific probiotic strain among other probiotics or members of the native microbiota is essential for strain selection and characterization, assessments of strain stability throughout the manufacturing process, for proper description of the material used in human intervention studies, for efficient tracking of the probiotic through the host, and for post-market surveillance including matching of strains isolated from any suspected infections.

Traditional methods used for detection of probiotics in the human GI tract include identification using colony morphology, fermentation patterns and combinations of these methods.

Unfortunately, the use of phenotypic tests or commercial identification systems such as API (Huys *et al.*, 2006; Vankerckhoven *et al.*, 2008), are inadequate for species level resolution. These systems (*e.g.* API 50 CHL) may be useful to obtain a first tentative classification at the genus level and fermentation type level, but the identification result should in any case be confirmed by other (molecular) methods (Vankerckhoven *et al.*, 2008).

Polyphasic identification uses a combination of genotypic and phenotypic approaches offering accurate identification and classification of closely related probiotic species (Vandamme *et al.*, 1996; Gancheva *et al.*, 1999).

Typing methods (such as pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA analysis (RAPD), amplified fragment length polymorphism (AFLP-PCR), repetitive DNA element (rep)-PCR and ribotyping) are primarily useful for differentiation of individual strains.

At present, the inappropriate use of identification methods is considered to be the major cause of mislabeling of probiotic products reported worldwide (Temmerman *et al.*, 2003; Masco *et al.*, 2005).

Reliable species identities of lactic acid bacteria are obtained by the use of pattern- and/or sequence-based molecular methods that provide sufficient experimental reproducibility and the proper taxonomic resolution. These methods make use of updated and easily available and validated identification databases (Vankerckhoven *et al.*, 2008).

Besides molecular DNA-based typing, extraction of whole-cell proteins, followed by sodium dodecyl sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE) separation, has been found to be a reliable and rapid way to characterize a large number of strains (Reuter *et al.*, 2002).

2.5.2.3. Safety of probiotics

Lactobacilli belong to the human normal microbiota of the GI and urogenital tracts. Moreover, lactobacilli have been used for many centuries in food fermentation processes and have a long history. Lactobacilli are generally regarded as safe (Borriello *et al.*, 2003). Therefore, the US Food and Drug Administration (FDA) has classified lactobacilli as GRAS (generally recognized as safe) organisms (Donohue, 2004). In Europe, the European Food Safety Authority (EFSA) has taken responsibility to launch the European initiative toward a "qualified presumption of safety" (QPS) concept which, similar to the GRAS system in the United States, is aimed to allow strains with an established history and safety status to enter the market without extensive testing requirements (EFSA, 2011b).

It is believed that the risk of infection with a probiotic *Lactobacillus* strain is similar to the risk of becoming infected with a *Lactobacillus* strain from the commensal microbiota (Ishibashi and Yamazaki, 2001). Endocarditis, bacteraemia and localised infections including abscesses caused by lactobacilli have been reported but mainly in individuals with severe underlying disease (Cannon *et al.*, 2005; Sanders *et al.*, 2010). On the other hand, specific strains of *Lactobacillus* probiotics have been administered to immunocompromised patients with HIV and no concerns have been reported (Salminen *et al.*, 2004). Other theoretical impact regarding the safety of putative probiotics include toxic or metabolic effects in the GI tract, transfer of antibiotic resistance to the GI microbiota (Snydman, 2008), and adverse effects mediated by immunomodulation (FAO/WHO, 2002; Sanders *et al.*, 2010).

In order to establish safety guidelines for probiotic organisms, recognizing that many are Generally Recognized as Safe, the FAO and WHO recommended that probiotic strains be characterized at a minimum with a series of tests: 1) determination of antibiotic resistance pattern, 2) assessment of certain metabolic activities (d-lactate production, bile salt deconjugation), 3) assessment of side effects in humans, 4) epidemiological surveillance of adverse incidents in consumers, 5) testing for toxin production, 6) testing for haemolytic activity, and 7) infectivity in immunocompromised animal models (FAO/WHO, 2002). Saarela *et al* (2000) suggested additional evaluation of the non-pathogenicity (no degradation of host mucins, no platelet aggregation properties) of putative probiotic strains.

The safety of probiotics should be confirmed in studies of humans. Although many research tools based on animal models or *in vitro* techniques are available, data from studies of humans are preferred whenever possible. Different

self-reported questionnaires for monitoring symptoms and adverse effects have been applied (Borriello *et al.*, 2003; Vankerckhoven *et al.*, 2008; Vlieger *et al.*, 2009). The tolerance and safe administration of probiotics could be evaluated by noninvasive measurements, such as measurements of body weight or blood pressure, parameters of hematologic analysis and of serum/plasma chemical analysis (liver and renal function indices).

Unfortunately, there is no generally accepted approach for safety assessment of probiotics, relying on differences *in vitro*, in animal experiments and in human intervention trials.

2.5.2.3.1. Antibiotic resistance of putative probiotics

There is concern about the antibiotic resistance of lactic acid bacteria being transferred to possibly pathogenic bacterial species, complicating the treatment of infection and leading to the spread of antibiotic-resistant bacteria (Mathur and Singh, 2005). Therefore, all probiotic products intended for use as feed or food additives must be examined to establish the susceptibility of the component strain(s) to a relevant range of antibiotics, using internationally recognised and standardised methods (Vankerckhoven *et al.*, 2008). There is a list of antibiotics, namely ampicillin, gentamicin, streptomycin, kanamycin, erythromycin, clindamycin, tetracycline, chloramphenicol, ciprofloxacin and quinupristin with dalfopristin, that the European Food Safety Authority (EFSA) has suggestsed for detecting the antibiotic susceptibility pattern of lactobacilli (EFSA, 2008). These antibiotics belong to a group of broad-spectrum antibiotics that are intended for treatment of gram-negative pathogens.

The presence of transmissible antibiotic resistance markers in the evaluation of strains is thus an important safety criterion. Today, 35 *Lactobacillus* species (including *L. acidophilus, L. gasseri*, *L. fermentum, L. paracasei* and *L. plantarum*) are considered to have QPS-status (Leuschner *et al.*, 2010; EFSA, 2011b). However, there exists a highly various range of species-specific natural antibiotic resistance among different species and strains of lactobacilli, mostly non-transmissible (Mändar *et al.*, 2001; Danielsen and Wind, 2003; Mikelsaar, 2011).

The knowledge of the resistance pattern of the probiotic would be useful to avoid using strains that carry transferable resistance genes. Plasmid-encoded erythromycin, tetracycline and chloramphenicol resistance has been reported in lactobacilli (Axelsson *et al.*, 1988; Lin *et al.*, 1996; Gevers *et al.*, 2003).

Some studies indicate a higher risk of antibiotic resistance transfer than previously believed, especially during treatment with antibiotics (Jacobsen *et al.*, 2007; Toomey *et al.*, 2009). On the contrary, Egervärn and co-workers (2010) have demonstrated that the tetracycline resistance gene from probiotic *Lactobacillus reuteri* was not transferable to bacteria in the GI tract of humans (Egervärn *et al.*, 2010).

In case there is detected an antibiotic resistance marker in a new probiotic *Lactobacillus* strain, it is necessary to confirm whether the antibiotic resistance is of chromosomal origin or it is carried by plasmids and is therefore putatively transferable (Saarela *et al.*, 2000; EFSA, 2008; Egervärn *et al.*, 2010). The latter plasmid should be deleted.

2.6. Functional properties and colonizing potential of probiotic lactic acid bacteria

The functional properties of probiotics include production of antimicrobial compounds, suppression of pathogens, expression of antioxidative activity, immune stimulation and colonizing potential such as tolerance to acid, bile and pancreatin (Saarela *et al.*, 2000; Mikelsaar and Zilmer, 2009). The functional requirements of probiotics should be established using *in vitro* methods and the results of these studies should be reflected in controlled human studies. The functional properties differ significantly among various *Lactobacillus* species and strains (Annuk *et al.*, 2003; Kõll *et al.*, 2008).

2.6.1. Acid, bile and pancreatin tolerance

To provide health benefits, *Lactobacillus* strains, which are mostly delivered in a food system, must overcome physical and chemical barriers in the GI tract, especially acid and bile stress (Del Piano *et al.*, 2006). Probiotics also need to possess the ability to survive in products with sufficient numbers during production and storage (Songisepp, 2005; Ljungh and Wadstrom, 2006).

In the stomach and the upper intestine, containing gastric acid and bile, bacterial survival under the adverse conditions is attributable primarily to their ability to maintain intracellular pH by means of proton-translocating ATPase (Bender and Marquis, 1987). Other mechanisms for survival include changes in the cell membrane, regulatory mechanisms, metabolic pathways, amino acid decarboxylation and heat-shock proteins (Lim *et al.*, 2000; Cotter and Hill, 2003).

2.6.2. Adhesion

The ability of lactobacilli to adhere to epithelial cells is considered an important feature in the process of colonization of the different human ecological niches such as the gut and the vagina. Adhesion of lactobacilli to epithelial cells is facilitated by several exported proteins (*e.g.* mucus binding protein, lectin-like mannose adhesin, sortase-dependent and other surface layer proteins), carbohydrates and lipoteichoic acid (Lebeer *et al.*, 2008; Sanchez *et al.*, 2008). A number of *in vitro* models (*e.g.* cell lines Caco-2 and HT-29, immobilised

intestinal mucus) have been used to study the adhesion properties of probiotic bacteria (Tuomola *et al.*, 1999; Ouwehand and Salminen, 2003; Laparra and Sanz, 2009).

The abilities of lactobacilli to adhere to the intestinal mucosa (*e.g.* cell lines) and the mucus layer may be different. Ouwehand and co-workers have shown that adherence of lactobacilli to the intestinal tissue was lower than to the intestinal mucus (Ouwehand *et al.*, 2002). Therefore, it has been suggested to use both test models for evaluation of the adhesive properties of lactobacilli. The auto-aggregative ability has also been used as an indicator for adhesion (Perez *et al.*, 1998; Collado *et al.*, 2008). In a previous study Annuk and coauthors showed the importance of the lectin profiles of different *Lactobacillus* sp. in autoaggregating strains, mainly of the *L. acidophilus* group (Annuk *et al.*, 2001).

In vitro results regarding the adherence capacities of lactobacilli are difficult to extrapolate to the GI tract situation *in vivo*, where individual host defense systems, competition with the resident microbiota for nutrients and space, mucosal shedding, and peristaltic flow, that continuously washes the GI tract epithelium, are likely to modify adhesion (Servin, 2004). There is a need for complex *in vitro* and colonization studies to assess the value of *in vitro* tests.

2.6.3. Antimicrobial activity

Lactobacilli produce a wide range of antibacterial compounds including sugar catabolites such as organic acids (*e.g.* lactic acid and acetic acid); oxygen metabolites such as hydrogen peroxide; fat and amino acid metabolites such as fatty acids, phenyllactic acid, and OH-phenyllactic acid; proteinaceous compounds such as bacteriocins, other low-molecular-mass peptides, and antifungal peptides/proteins and particular compounds such as reuterin and reutericyclin (Ouwehand and Vesterlund, 2004).

Bacteriocins are most often active towards closely related gram-positive bacteria, while the producer cells are tolerant to their own bacteriocins (De Vuyst and Leroy, 2007). However, activity against gram-negative bacteria, *e.g. Salmonella,* by *Lactobacillus* bacteriocins or bacteriocin-like substances has also been described (Lin *et al.*, 2008). The most common mode of action of antimicrobial compounds is the formation of pores in the bacterial membrane, but they can also act by prevention of cell-wall synthesis, inhibition of RNA synthesis and inhibition of bacterial phospholipases (Sang and Blecha, 2008).

Production of plantaricins is typical of several *L. plantarum* strains. Subclass IIa includes food-borne pathogens inhibiting bacteriocins, sub-class IIb harbours di-peptide bacteriocins of a wide activity range: plantaricin EF and plantaricin JK, also plantaricin S (Maldonado *et al.*, 2002).

2.6.4. Ability to persist in the gut

When selecting potential probiotic strains, there arises the question about which probiotic strains will survive and transiently colonize the GI tract. Many strains currently used as probiotics have a documented ability to survive in the human gut and are recovered alive in faeces (Goldin *et al.*, 1992; Mätto *et al.*, 2006; Tuohy *et al.*, 2007).

The isolation of the same strain over time after cessation of consumption could then imply persistent colonization. It has been demonstrated in clinical trials that probiotic lactobacilli persist in faecal samples for a few days up to a week after intake has stopped (Goldin *et al.*, 1992; Saxelin *et al.*, 1995; Jacobsen *et al.*, 1999; Mätto *et al.*, 2006).

2.6.5. Antioxidative properties

Lactic acid bacteria (including lactobacilli and bifidobacteria) have demonstrated antioxidative ability *in vitro* experiments (Kaizu *et al.*, 1993; Kullisaar *et al.*, 2002; Koller *et al.*, 2008; Kaushik *et al.*, 2009).

The antioxidative properties of lactic acid bacteria include ROS inactivation via enzymatic mechanisms (*e.g.* by a coupled NADH oxidase/reductase system, superoxide dismutase) and non-enzymatic mechanisms such as scavenging by glutathione system (Kaizu *et al.*, 1993; Kullisaar *et al.*, 2002; Lee *et al.*, 2005). The probiotic strain *L. fermentum* ME-3 contains both glutathione peroxidase and glutathione reductase by which the strain can transport GSH from the environment and even synthesize it (Kullisaar *et al.*, 2010). GSH is the cellular redox buffer acting as a scavenger of free radicals and toxic substances, and serving as a co-substrate for detoxification enzymes (Hansen *et al.*, 2009).

In a mouse model of *Salmonella* Typhimurium infection, the administered antioxidative *L. fermentum* ME-3 increased total antioxidative activity and the glutathione redox value in the intestinal mucosa, which indicates a manifestation of antioxidative potential *in vivo* (Truusalu *et al.*, 2004; Truusalu *et al.*, 2008; Truusalu *et al.*, 2010).

A recent study showed that probiotic treatment (*L. plantarum* HEAL19) with polyphenol rich fruit (bilberry) reduced lipid peroxidation in a mouse model of intestinal ischemia-reperfusion (Jakesevic *et al.*, 2011).

2.7. Assessment of the safety and efficacy of probiotics in clinical trials

The most important proof of probiotic safety and functional efficacy should be tested with clinical studies in children, adults and elderly. Intervention studies provide stronger evidence than observational studies. Clinical intervention trials for probiotic evaluation are divided into four phases: phase 1 (safety), phase 2

(efficacy), phase 3 (effectiveness) and phase 4 (surveillance) (FAO/WHO, 2002; Reid *et al.*, 2003).

First, phase 1 pilot clinical trials on healthy volunteers to exclude the adverse effects of probiotic administration on gut health, and on the biochemical and cellular indices of blood, reflecting the proper functions of human organs, should be conducted (Reid, 2005; Rijkers *et al.*, 2010).

It has been suggested, from the healthy human's perspective, that evaluation of tolerance and safety may be based on self-reported questionnaires for monitoring symptoms and adverse effects, blood safety parameters and number and type of adverse events (Borriello *et al.,* 2003; Vankerckhoven *et al.,* 2008; Vlieger *et al.,* 2009; Wind *et al.,* 2010). The tolerance and safe administration of probiotics can be evaluated by several measurements, such as measurement of body weight or blood pressure, and parameters of haematologic analysis and of serum/plasma chemical analysis (liver and renal function indices).

Next, in efficacy studies (phase 2) the manifestation of the strain's functional properties, either by improving some physiological functions of the host (*e.g.* antimicrobial, metabolic, immunogenic, antioxidative) or by reducing the risk of some diseases after the consumption of the probiotic product in large groups of volunteers, should be tested.

There are several designs for probiotic intervention studies: open-label trial, double-blind, placebo-controlled trial; and randomised, double-blind, placebo-controlled crossover trial. Clinical trials for evaluation of the efficacy of probiotics should be randomised and double-blinded.

For elucidation of the mechanisms of probiotic impact, different study designs including non-randomised double-blind placebo controlled or open-label prospective and cross-sectional studies are also suggested (EFSA, 2011c).

2.7.1. Efficacy of antioxidative probiotics in human studies

Quite a new area of research is the response of host cells and molecules such as lipid, proteins and DNA to oxidative damage. Up to now it has caused several misunderstandings in evaluation of the clinical efficacy of antioxidative substances. In our previous studies several markers of oxidative stress and antioxidative defence were applied. Namely, the consumption of the probiotic *L. fermentum* ME-3 in fermented goat milk or in probiotic capsules increased TAS values in healthy subjects (Kullisaar *et al.*, 2003; Songisepp *et al.*, 2005). Also, in patients with mild-to-moderate atopic dermatitis the consumption of *L. fermentum* ME-3 goat milk improved blood antioxidative activity (TAS and TAA), GSH levels increased and the ratio GSSG/GSH decreased (Kaur *et al.*, 2008).

Recently, the Nutrition, Dietetic and Allergy (NDA) panel was asked by the European Food Safety Authority (EFSA) to draft guidance on scientific requirements for health claims related to antioxidants, oxidative damage and cardiovascular health. The document was not intended to include an exhaustive

list of beneficial effects and studies/outcome measures which are acceptable (EFSA, 2011a). However, the panel doubted that the overall antioxidant capacity of plasma could exert a beneficial effect in humans as required by the EC Regulation 1924/2006. Further, they confirmed that the scientific substantiation of health claims on the protection of molecules from oxidative damage required at least one appropriate marker of oxidative modification of the target molecule assessed in human studies, preferably in combination with other marker(s) determined in the same study.

Measurements of oxidative damage to lipids (*i.e.* lipid peroxidation) can be obtained *in vivo* by measuring oxidised LDL particles in blood. Several other proposed methods, include lipid peroxides, HDL-associated paraoxonases and conjugated dienes, could be used as supportive evidence if used in the same study.

Concerning protection from oxidative damage, there are no clinical intervention trials studying the impact of antioxidative lactic acid bacteria combined with a prebiotic on host serum and on cell antioxidative activity, particularly, on the amount and state of lipid molecules in healthy volunteers.

3. THE AIMS OF THE STUDY

The general goals of the research were:

- to evaluate the functional properties of some commercial probiotics;
- to compare the required properties of probiotic strains obtained in *in vitro* and animal experiments with their impact on the health indices of the human organism after consumption.

The aims of the present study were:

1) To evaluate the *in vitro* antagonistic activity of some commercial probiotic *Lactobacillus* and *Bifidobacterium* strains against various gastric, entero- and urinary pathogens residing in distinct niches throughout the GI tract and to assess the antioxidative activity of the above mentioned probiotic strains.
2) To establish the efficacy of consumption of a synbiotic product containing selected commercial antimicrobial and antioxidative strains (*L. fermentum* ME-3, *L. paracasei* 8700:2, *B. longum* 46) and raftilose P95 in lowering the oxidative stress markers of blood (oxLDL, BDC-LDL) in healthy persons.
3) To screen the putative probiotic *Lactobacillus* strains of the intestinal tract, deposited in the Human Microbiota Biobank of the Department of Microbiology of the University of Tartu, for their colonizing potential using *in vitro* tests mimicking the GI tract conditions.
4) To assess the safety of the probiotic *L. plantarum* TENSIA (DSM 21380) and of previously selected six *Lactobacillus* strains using *in vitro* and animal experiments.
5) To evaluate the survival and persistence of selected putative probiotic candidates and of the probiotic strain TENSIA in the GI tract of healthy volunteers after consumption.
6) To assess the safety of consumption of the probiotic strain *L. plantarum* TENSIA and five capsulated putative probiotic strains in healthy volunteers.

4. MATERIAL AND METHODS

An overview of the material and methods used in this study is presented in Table 2 and, additionally, a detailed description is available in the following section.

Table 2. Study subjects, experimental animals, microbial strains and performed investigations.

Study subjects / Objectives	**Study design and description**	**Presented in papers**
a) Probiotic strains *L. rhamnosus* GG (ATCC 53103) *L. fermentum* ME-3 (DSM 14241) *L. acidophilus* La5 *L. plantarum* 299v (DSM 9843) *L. paracasei* 8700:2 (DSM 13434) *B. lactis* Bb12 *B. longum* 46 (DSM 14583) b) pathogenic bacteria *E. coli* (ATCC 700336, pyelonephritic strain) *E. coli* (ATCC 700414, cystitic strain) *Salmonella enterica* ssp. *enterica* (ATCC 13076) *Shigella sonnei* (ATCC 25931) *H. pylori* (NCTC 11637) *C. difficile* (VPI 10463)	*In vitro* assessment of functional properties (antagonistic and antioxidative activity) of commercial probiotic lactobacilli and bifidobacteria	I
53 healthy adult volunteers: 41 female + 12 male, *H. pylori*-positive (n=28) and *H. pylori*-negative persons (n=25)	RDBPCCOT (Efficacy study) Evaluation of antioxidative activity	II, present study
Selected 6 putative probiotic *Lactobacillus* strains from Biobank *L. gasseri* 177 *L. gasseri* E16B7 *L. acidophilus* 821-3 *L. paracasei* 317 *L. fermentum* 338-1-1 *L. paracasei* 1-4-2A Probiotic *L. plantarum* TENSIA (DSM 21380)	*In vitro* screening of colonizing potential (auto-aggregation ability, acid, bile and pancreatin tolerance) and safety *in vitro*	III
Pathogens (controls for haemolytic activity testing) *Staphylococcus aureus* (ATCC 25923) *Streptococcus pyogenes* (ATCC 19615) *Streptococcus pyogenes* (human clinical isolate)	Testing of haemolytic activity of lactobacilli	III

Table 2. Continuation

Study subjects / Objectives	Study design and description	Presented in papers
BALBc mice (n=10) / NIH mice (n=10)	Safety assessment *in vivo*	III, V
15 healthy adult volunteers (9 female + 6 male)	OLT Assessment of survival/persistence and safety of the selected 5 strains in human trial	IV
18 healthy over 60 year-old persons (17 female + 1 male)	DBPCT Assessment of survival/persistence and safety of *L. plantarum* TENSIA	V, VI
12 healthy adult volunteers (7 female + 5 male)	DBPCT Assessment of survival/persistence and safety of *L. plantarum* TENSIA	V, VI

DBPCT – double-blind, placebo-controlled trial; RDBPCCOT – randomised, double-blind, placebo-controlled crossover trial; OLT – open-label trial

Altogether 98 healthy adults without known health problems completed the clinical trials of this study.

Commercially available probiotic *Lactobacillus* sp (n=5) and *Bifidobacterium* sp (n=2) strains, intestinal isolates of different species (n=6) of Estonian and Swedish 1–2 yr old infants, a patented *Lactobacillus* strain (*Lactobacillus plantarum* TENSIA, DSM 21380), and 9 pathogenic reference strains were used *in vitro* experiments.

4.1. Microbial strains tested *in vitro*

4.1.1. Probiotic lactobacilli and bifidobacteria strains (Papers I, II)

The following well-known probiotic bacteria (Figure 2) were selected: *L. rhamnosus* GG (ATCC 53103; Valio, Helsinki, Finland), *L. fermentum* ME-3 (DSM 14241, University of Tartu, Estonia), *L. acidophilus* La5 (Chr. Hansen, Hørsholm, Denmark), *L. plantarum* 299v (DSM 9843, Probi, Lund, Sweden), *L. paracasei* 8700:2 (DSM 13434; Probi, Lund, Sweden), *B. lactis* Bb12 (Chr. Hansen, Hørsholm, Denmark) and *B. longum* 46 (DSM 14583, University of Turku, Finland) were used in the present study (Table 2, paper I). These six commercial probiotic strains (except *L. fermentum* ME-3) were obtained from the culture collection of the University of Turku (partner of the 5th Framework Programs "Microfunction").

Lactobacilli strains were grown on MRS agar and bifidobacteria were grown on Wilkins-Chalgren agar with 7% horse blood. The strains were Gram stained and examined with light microscopy for cell morphology under 1000X magnification to reveal differences in cell morphology (Figure 2a). Molecular fingerprints of the *Lactobacillus* strains are depicted in Figure 2b. Species identification was confirmed by comparison of the 16S-23S rRNA gene spacer regions with those of the reference strains.

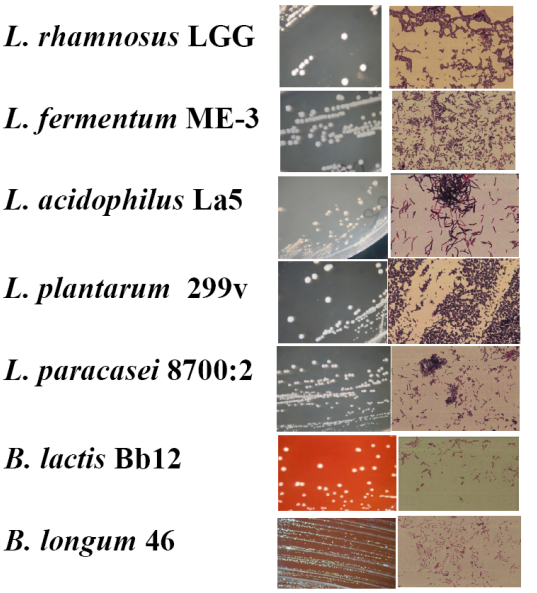
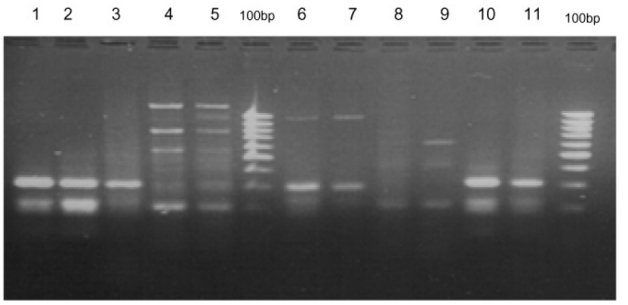


Figure 2a. Colony and cell morphology of the tested commercial probiotic strains.



1, *L. paracasei* 8700:2; *L. paracasei* spp *paracasei* DSM 20020; 3, *L. paracasei* DSM 5622; 4, *L. plantarum* 299v; 5, *L. plantarum* ATCC 14917; 6, *L. acidophilus* La5; 7, *L. acidophilus* ATCC 4356; 8, *L. fermentum* ME-3; 9, *L. fermentum* ATCC 14931; 10, *L. rhamnosus* GG; 11, *L. rhamnosus* ATCC 14931; 100 bp DNA ladder.
The *Lactobacillus* species-specific ITS-PCR was carried out by using primers 16S-1500F and 23S-32R (DNA Technology AS) targeted on the 16S-23S rRNA intergenic spacer region.

Figure 2b. Molecular fingerprints of commercial *Lactobacillus* strains. Detected by applying ITS-PCR (Štšepetova *et al*., 2002).

4.1.2. Putative probiotic strains
(Papers III, IV)

Hundreds of *Lactobacillus* strains were isolated from faecal samples of 1 to 2-year-old Estonian and Swedish children in a joint Estonian-Swedish project for evaluation of development of allergy (Mikelsaar *et al.*, 2002). In the present study six strains were used according to the results of *in vitro* screening (colonizing potential, potential of haemolytic activity and antibiotic susceptibility patterns).

4.1.3. Probiotic *L. plantarum* TENSIA
(Papers III, V, VI)

Lactobacillus plantarum TENSIA was isolated from a faecal sample of a healthy Estonian child during a comparative study of the microbiota of Estonian and Swedish children (Mikelsaar *et al.,* 2002). *L. plantarum* TENSIA belongs to the Bio-Competence Centre of Healthy Dairy Products LCC, Estonia. *L. plantarum* TENSIA is deposited in Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH-DSMZ under number DSM 21380. Probiotic cheese comprising *L. plantarum* TENSIA in semi-hard Edam-type cheese has been developed in cooperation with the Dairy Cooperative E-Piim. The *L. plantarum* TENSIA maintained good viability in probiotic cheese (Rätsep *et al.*, 2009).

4.1.4. Pathogenic strains
(Papers I, III)

The pathogenic target bacteria *E. coli* ATCC 700336 (pyelonephritic strain), *E. coli* ATCC 700414 (cystitic strain), *Salmonella enterica* ssp. *enterica* ATCC 13076, *Shigella sonnei* ATCC 25931, *H. pylori* NCTC 11637, *C. difficile* VPI 10463 were used as the target bacteria in the antagonistic activity experiments (Table 2).

The gram-positive cocci *Staphylococcus aureus* (ATCC 25923) and two *Streptococcus pyogenes* strains (ATCC 19615 and a human clinical isolate) were used as the positive controls in testing haemolytic activity of putative probiotic bacteria.

4.2. Characterization of putative probiotic strains (Paper III)

4.2.1. Testing of auto-aggregation ability

The auto-aggregation ability of putative probiotic lactobacilli and of the probiotic *L. plantarum* TENSIA was determined as described by Pascual *et al.* (Pascual *et al.*, 2008) with certain modifications. Briefly, lactobacilli were grown for 48h at 37°C on MRS agar (Oxoid, Basingstoke, United Kingdom) plates in a microaerobic environment (10% CO_2). A loopful (10 µl) of culture was suspended on a glass microscope slide in 1 ml of 0.9% saline solution (pH 6.7) to a final concentration corresponded to McFarland Nephelometer Standard 3. Auto-aggregation was determined as the ability to form aggregates (clearly visible sand-like particles) within 2 min at room temperature. The results were expressed as: score 0 – no auto-aggregation, score 1 – intermediate auto-aggregation (presence of some flakes), and score 2 – strong auto-aggregation.

4.2.2. Testing of acid, bile and pancreatin tolerance (Paper III)

The effect of low pH, bile, and pancreatin on the survival of lactobacilli was examined in microwell plates (Costar® 96 Well Cell Culture Clusters, Myriad Industries, San Diego, CA). MRS broth (Oxoid) was adjusted to a pH range between pH 5.0 and pH 2.0 to test acid tolerance and contained oxgall (2% w/v) (Sigma, Steinheim, Germany) and/or pancreatin (0.5% w/v) (Sigma, Steinheim, Germany) to test bile and pancreatin tolerance. Each 180-µl volume of adjusted and nonadjusted MRS broth (as control; pH 6.0) was inoculated with 20 µl lactobacilli suspension (McFarland 1.0 turbidity standard) and incubated in a microaerobic environment at 37 °C for 4 h.

The number of cells in the suspension of lactobacilli and the number of surviving cells following incubation in pH-, bile- and pancreatin-adjusted media was determined by plating 100 µl of tenfold serially diluted sample onto MRS agar (Jacobsen *et al.*, 1999; Kõll *et al.*, 2008). Strains with viable cell counts equal to viable counts before incubation in pH-, bile- and pancreatin adjusted media were considered as resistant to a particular pH, bile and pancreatin concentration.

The colonizing potential (auto-aggregation, tolerance to acid, bile and pancreatin) of putative probiotics were determined by Piret Kõll.

4.2.3. Testing of haemolytic activity
(Paper III)

A single line of lactobacilli culture (grown in MRS broth for 48h) was streaked onto blood agar plates containing either human or horse blood. The haemolytic activity of putative probiotics was verified visually after 24h and 48h of incubation in aerobic, microaerobic (10% CO_2) and anaerobic (90% N_2, 5% CO_2, 5% H_2) environments. The blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green halo around colonies) or γ-haemolysis (no zones around colonies) (Pfaller *et al.*, 2007).

The *Staphylococcus aureus* strain (ATCC 25923) and two *Streptococcus pyogenes* strains (ATCC 19615 and a human clinical isolate, respectively) were used as the positive controls.

4.2.4. Testing of antibiotic susceptibility
(Papers III, V, VI)

Minimum inhibitory concentrations (MICs) of ampicillin, vancomycin, gentamicin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin, rifampicin and trimethoprim-sulfamethoxazole were determined by the E-test method as described by the manufacturer.

Wilkins-Chalgren (Oxoid, Basingstoke, United Kingdom) agar plates with 5% horse blood, E-test antibiotic strips (AB Biodisk, Solna, Sweden) and 48h of incubation at 37ºC in an anaerobic glove chamber were used. The MIC (minimal inhibitory concentration) was determined according to the Clinical and Laboratory Standards Institute (CLSI) (former National Committee for Clinical Laboratory Standards (NCCLS)) guidelines (Jorgensen and Turnidge, 2003; CLSI, 2006).

Strains with MICs equal and higher than the breakpoints (μg/ml) were considered as resistant. More recently, EFSA proposed microbial breakpoints for lactobacilli at the genus and species levels, and for different fermentation types (OHOL, FHEL, OHEL) of lactobacilli (EFSA, 2008).

4.3. Total anti-oxidative activity
(Paper I)

The total antioxidative activity (TAA) and the total antioxidative status (TAS) of seven commercial probiotic bacteria were assessed. Testing of the anti-oxidative activity of the bacteria was performed by Tiiu Kullisaar from the Department of Biochemistry.

TAA was measured using the linolenic acid test (LA-test) (Kullisaar *et al.*, 2002). Lactobacilli were grown in MRS broth under microaerobic conditions

and bifidobacteria in MRS broth supplemented with cysteine in an anaerobic environment for 48h.

Probiotic bacteria were pelleted by centrifugation (10 000 *g* for 10 min), washed twice and re-suspended in isotonic saline at 4°C. The density of the suspension was adjusted to 10^9 CFU/ml using an absorbance of 1.1 at 600 nm. TAA was expressed as the percentage of inhibition of the peroxidation of the linolenic acid standard by the sample, which predominantly reflects the anti-oxidative status of the lipid fraction.

The TAS of the bacteria was assessed using a commercially available kit (TAS, Randox Laboratories Ltd. Ardmore, UK) (Kullisaar *et al.*, 2003). A water-soluble vitamin E (Trolox) served as the standard. This method is based on inhibition of the absorbance of the ferrylmyoglobin radicals of 2,2'-azinobis-ethylbenzothiazoline 6-sulfonate (ABTS+) generated by the activation of metmyoglobin peroxidise with H_2O_2, and indicates the anti-oxidative activity in water fractions.

The total anti-oxidative values of the probiotic bacteria were considered high if TAA was >20% and TAS > 0.1 mmol/l.

4.4. Antagonistic activities of commercial probiotics (Paper I)

The antagonistic activity of commercial probiotic strains (*L. rhamnosus* GG, *L. fermentum* ME-3, *L. acidophilus* La5, *L. plantarum* 299v, *L. paracasei* 8700:2, *B. lactis* Bb12, *B. longum* 46) against entero- and uropathogens was assessed using solid and liquid media under microaerobic and anaerobic conditions.

The antimicrobial activity of the probiotic strain *L. plantarum* TENSIA against entero- and uropathogens was detected in microaerobic conditions on solid media.

4.4.1. Antimicrobial activity of lactobacilli and bifidobacteria on agar plates

Briefly, prior to final inoculation, the lactobacilli were pre-cultivated three times in the respective environment. Bifidobacteria, as strict anaerobes, were grown only in an anaerobic environment.

The antimicrobial activity of probiotic lactobacilli and bifidobacteria against selected target bacteria was assessed using a streak line method. The following media were used: modified MRS medium (MRS medium without tri-ammonium-citrate and sodium-acetate; pH 7.2) for lactobacilli, Wilkins-Chalgren agar with 7% horse blood for bifidobacteria and *C. difficile*, and Columbia Agar Base supplemented with 7% horse blood and 1% Vitox for *H. pylori*.

Lactobacilli and bifidobacteria were seeded in the middle of the agar plate using a 10 μl loop. Following the incubation in a microaerobic/anaerobic environment at 37°C for 48h for lactobacilli, or in an anaerobic environment for bifidobacteria, growth was inactivated with chloroform gas which was used for 2h. The following target bacteria were tested: *E. coli, Salmonella enterica* subspecies *enterica* and *S. sonnei* grown in peptone water for 18h (turbidity 10^9 CFU/ml), a *H. pylori* suspension in Brucella broth (BBL, Cockeysville, MD, USA) adjusted to McFarland density of 3–4, and a *C. difficile* suspension in saline adjusted to a McFarland density of 1. The target bacteria were seeded using a 1 μl loop, in duplicate, perpendicular to the streak line of lactobacilli and bifidobacteria on the respective media as described above, and were incubated at 37°C. *E. coli, Salmonella enterica* subspecies *enterica* and *S. sonnei* were incubated in aerobic environment for 18h, *H. pylori* was grown in a microaerobic environment for 3 days, and *C. difficile* was incubated anaerobically for 2 days.

The antagonistic activity of lactobacilli and bifidobacteria was estimated as the width of the inhibition zone (mm) of the target bacteria extending from the culture line of probiotic bacteria (Mikelsaar *et al.*, 1987; Annuk *et al.*, 2003). The inhibitory effect of the lactobacilli and bifidobacteria was ranked as high (>25 mm), moderate (13–25 mm) and low (0–12 mm).

4.4.2. Antimicrobial activity of probiotic lactobacilli and bifidobacteria in broth

Probiotic bacteria were pre-incubated as described above. The strains of *E. coli*, *Salmonella enterica* subspecies *enterica* or *S. sonnei* in equal aliquots were co-incubated with the suspensions of lactobacilli or bifidobacteria in isotonic saline (10^9 CFU/ml) at 37°C for 24h under microaerobic and/or anaerobic conditions. Thereafter, the number of colony forming units (CFU/ml) of the target gram-negative bacteria was semi-quantitatively determined on peptone agar (Gould, 1965).

Inhibition of pathogen growth was calculated by subtracting the number of target bacteria remaining in the co-incubation tube from the number in the control tube with only target bacteria (Annuk *et al.*, 2003). The result was expressed as $\log_{10}$ CFU/ml. The values of inhibition of growth of pathogens by probiotics in liquid media were ranked as high, moderate and low (decrease by 5.9–6.5, 3.4–5.8 and 0.6–3.2 $\log_{10}$ CFU/ml, respectively).

4.4.3. Antagonistic activity between probiotic strains and putative probiotic lactobacilli strains

The antimicrobial activity of probiotic lactobacilli, putative probiotic lactobacilli and probiotic bifidobacteria against selected target lactobacilli or

bifidobacteria was assessed using a streak line method as described above (present study).

Briefly, lactobacilli were cultivated in microaerobic condition and bifidobacteria, as strict anaerobes, were grown only in an anaerobic environment. The following media were used: modified MRS medium (MRS medium without triammonium-citrate and sodium-acetate; pH 7.2) for lactobacilli, Wilkins-Chalgren agar with 7% horse blood for bifidobacteria. Lactobacilli and bifidobacteria were seeded in the middle of the agar plate using a 10 μl loop. Following the incubation in a microaerobic/anaerobic environment at 37°C for 48h for lactobacilli, or in an anaerobic environment for bifidobacteria, growth was inactivated with chloroform gas during 2h. The target bacteria (lactobacilli or bifidobacteria) were seeded using a 1 μl loop, in duplicate, perpendicular to the streak line of lactobacilli and bifidobacteria on the respective media and environment as described above, and were incubated at 37°C for 2 days.

The antagonistic activity of lactobacilli and bifidobacteria was estimated as the width of the inhibition zone (mm) of the target bacteria extending from the culture line of the bacteria (Mikelsaar *et al.*, 1987; Annuk *et al.*, 2003).

4.5. Determination of organic acids
(Papers I, V)

The production of organic acids (acetic acid, lactic acid, succinic acid) of probiotic strains (*L. rhamnosus* GG, *L. fermentum* ME-3, *L. acidophilus* La5, *L. plantarum* 299v, *L. paracasei* 8700:2, *B. lactis* Bb12, *B. longum* 46) and of the probiotic *L. plantarum* TENSIA was performed using gas chromatographic analyses in cooperation with Ms Jelena Stsepetova.

The production of organic acids was estimated by gas chromatography as described by Holdeman *et al.* (1977). The gas chromatograph (Hewlett-Packard model 6890) was equipped with a hydrogen flame ionization detector and an auto sampler (model 7683). The HP Chemical Station for the GC System (A.06 revision) was used. Analyses were performed following the cultivation of lactobacilli in microaerobic and anaerobic environments in modified MRS broth for 24h and cultivation of bifidobacteria under anaerobic conditions in MRS broth supplemented with cysteine for 24h.

4.6. Faecal recovery of *Lactobacillus* strains
(Papers IV, V)

The survival and persistence of consumed putative probiotic strains and the probiotic *L. plantarum* TENSIA were investigated in faecal samples of healthy adults and healthy persons over 60 years of age. The impact of the consumption

of the above mentioned strains on the counts of indigenous lactobacilli was also measured.

Survival of *Lactobacillus* strains indicates their ability to survive passage through the GI tract during consumption. Persistence shows their ability to maintain viability in the GI tract after discontinuation of consumption of the probiotic product.

4.6.1. Isolation and preliminary identification of *Lactobacillus* spp

The counts of total faecal *Lactobacillus* isolates of indigenous lactobacilli, and of the *Lactobacillus* strains consumed during the trials were evaluated in faecal samples by a conventional cultivation method. Serial dilutions (10^{-2}– 10^{-9}) of weighed faecal samples were prepared with sterile saline and 0.05 ml aliquots was seeded onto MRS and Rogosa agar media (Mikelsaar *et al.*, 2002; Songisepp *et al.*, 2005). The plates were incubated at 37°C for 3 days microaerobically in a 10% CO_2 environment (incubator IG 150, Jouan, France).

Provisional identification of lactobacilli isolates was based on gram-positive rod-shaped nonsporing cell morphology and negative catalase reaction. Further identification included biochemical characteristics and/or API 50 CHL (BioMérieux, Marcy-l'Etoile, France). Isolates were provisionally identified as lactobacilli and were further analysed for fermentation type. The ability of isolates to grow in MRS broth for 24h in a 10% CO_2 environment at 15°C and 37°C and to produce gas in MRS agar with 1% glucose was also assessed. The fermentation of glucose without gas production, growth at 37°C and no growth at 15°C identifies obligate homofermentative lactobacilli (OHOL); growth both at 15°C and 37°C without gas production is characteristic of facultative heterofermentative lactobacilli (FHEL), whereas gas production at 37°C and variable growth at 15°C are characteristic of obligate heterofermentative lactobacilli (OHEL). The count of *Lactobacillus* species was expressed in $\log_{10}$ colony forming units (CFU) per gram of faeces ($\log_{10}$ CFU/g). The detection level of lactobacilli was 3.0 $\log_{10}$ CFU/g faeces. Lactobacilli were considered indigenous if they were different from consumed strains according to molecular typing results.

4.6.2. Detection of survival and persistence of consumed putative probiotic strains

The intestinal survival and persistence of the probiotic TENSIA were detected in faecal samples of 30 healthy volunteers after consumption of probiotic cheese. The intestinal survival and persistence of 4 putative probiotic strains, *L. gasseri* 177, *L. gasseri* E16B7, *L. paracasei* 317 and *L. fermentum* 338-1-1, were investigated in 9 persons and the intestinal survival and persistence of the

selected strain *L. acidophilus* 821-3, in 14 persons after consumption of probiotic capsules.

The intestinal survival and persistence of ingested *L. plantarum* TENSIA were detected by conventional cultivation and further typing with specific primers for arbitrarily primed PCR (AP-PCR). 5 putative probiotic strains (177, E16B7, 317, 338-1-1, 821-3) consumed in a mixture were identified applying the same methods. In addition, for detection of the persistence of the *L. acidophilus* 821-3 a strain specific real-time PCR was developed in collaboration with the Professor Lennart Hammarström's group, Department of Laboratory Medicine, Karolinska Institute, Sweden.

4.6.2.1. Molecular methods

4.6.2.1.1. AP-PCR typing

Putative *Lactobacillus* isolates were typed by AP-PCR. Pure cultures were cultivated on MRS agar microaerobically for 24h at 37°C in 10% CO_2. Genomic DNA was extracted with the QIAamp DNA Mini Kit 50 (QIAGEN GmbH., Hilden, Germany) according to the manufacturers' instructions. AP-PCR typing was performed using three primers: ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3'), ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') and Hayford primer (5'-ACGCGCCCT-3') (DNA Technology A/S, Aarhus, Denmark) as described previously (Zhong *et al.*, 1998; Alander *et al.*, 1999; Hayford *et al.*, 1999).

The PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide (0.1 µl/ml) in the Tris/acetic acid/EDTA (TAE) electrophoresis buffer (Bio-Rad Laboratories, Hercules, USA) at a constant voltage of 100 V. A 1 kb ladder (Fermentas, Vilnius, Lithuania) was used as the marker. The banding patterns of the isolates were visualized with UV light and compared with those of the consumed *Lactobacillus* strains.

4.6.2.1.2. PCR-DGGE

The DNA of faecal samples was extracted using QIAamp DNA Stool Mini Kit (QIAGEN GmbH., Hilden, Germany). The amount of DNA was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide. To investigate the microbiota of the *Lactobacillus*-specific GI tract PCR was performed using the 16S rDNA primers Univ-515r-GC (5'-CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGATCGTATTACC GCGGCTGGCA-3') and Lab-0159f (5'-GGAAACAGRTGCTAATACCG-3'). Nested PCR was performed with these primers on previously generated products from amplification with 7f (AGAGTTTGATCTACTGGCTCAG) and Lab677-r (5'- CACCGCTACACATGGAG-3') (Heilig *et al.*, 2002).

PCR-DGGE and AP-PCR were performed by Jelena Stsepetova.

4.6.2.1.3. Real-time PCR

Total genomic DNA from faecal samples was extracted by the QIAamp DNA Stool Minikit (QIAGEN GmbH., Hilden, Germany). Quantification of the *L. acidophilus* 821-3 strain, the *L. acidophilus* species and total lactobacilli in faecal samples was performed by real-time PCR using ABI Prism 7300 (Applied Biosystems, USA). The detailed experimental procedure, protocol, strain-specific primers and a Taqman-MGB are described in paper IV.

Quantification of the *L. plantarum* TENSIA strain in faecal samples was performed by real-time PCR using ABI Prism 7500 (Applied Biosystems, USA). This analysis was done by Eerik Jõgi from the Institute of Technology of the University of Tartu. In order to identify a specific genomic sequence for *L. plantarum* TENSIA, oligonucleotides were designed between the conserved regions (114415-115632) of the *L. plantarum* strain WCFS1 complete genome (Genbank accession no. AL935255). Using *L. plantarum* TENSIA genomic DNA as the template and the primers tensia1 (5'-GTTAAGGTTTGCAAC-AGGTC-3') and tensia2 (5'-GACAATACTAGCCCAAGCTG-3') 750bp PCR products were amplified.

Amplified fragments were cloned with InsTACloneTM PCR Cloning Kit (Fermentas, Vilnius, Lithuania) to pUC57/T vector and transformed to competent *E. coli* DH5α cells. Clones with inserts were sequenced (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). The sequenced 750 bp insert (plasmid pUC57/T TEN) a contained partial sequence of the phospho-2-dehydro-3-deoxyheptonate aldolase gene. This sequence was used as the target for real-time PCR primers.

The primers for real-time PCR were designed with the web-based program Primer3 (http://frodo.wi.mit.edu/primer3). The resulting primers tensiaRT31 (5'-AACGCAAGCTTTATCCGATG-3') and tensia RT32 (5'-TGTTAAGGTT-TGCAACAGGTCA-3') reaction conditions were tested by regular PCR. The specificity of primers was tested by PCR using 6 different *L. plantarum* strains. The real-time PCR reaction mixture (25 µl) contained 5 µl 5xHOT FIREPol® EvaGreen® qPCR mix (high ROX) (Solis BioDyne, Tartu, Estonia), 20 pmol (each) specific primers and 2.5 µl of template DNA. The amplification program consisted of one cycle of 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 60°C for 60 s. The fluorescent product was detected at the last step of each cycle. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.2°C/s increments from 60 to 99°C, with continuous fluorescence collection. Standard curves were obtained using ten-fold dilutions of plasmid pUC57/T TEN containing the corresponding amplicon ranging from 10^5 to 10 copies.

4.7. Safety testing of putative probiotics in animal models
(Papers III, V, VI)

In order to determine the safety of consumption of the probiotic *L. plantarum* TENSIA and six putative probiotic lactobacilli *in vivo*, two animal trials were performed in collaboration with Kai Truusalu and Imbi Smidt.

To determine the safety of the probiotic *L. plantarum* TENSIA, 10 NIH line mice were given the commercial diet R-70 (Lactamin, Sweden) and tap water *ad libitum* and the amount of 50 g of cheese was added per cage daily. The average consumption of cheese per mouse per day was calculated after the leftover cheese was weighed. Ten mice out of twenty belonged to the test group fed with probiotic cheese containing *L. plantarum* TENSIA in concentration of 9.6 $\log_{10}$ CFU/g for 30 consecutive days. The control group was fed with the same amount of regular cheese without added lactobacilli. Faeces for lactobacilli counts were sampled on days 0, 3, 10, 15, 20 and 30. At the end of the trial autopsy was performed. The mice were sacrificed by cervical dislocation on day 30 and samples for microbiological analyses were collected. Heart blood (10 µl), homogenized tissue of the liver, and spleen were tested for possible translocation of the gut microbiota. For detection of *L. plantarum* TENSIA, MRS and blood agar were used. The samples of the small intestine and large intestine for lactobacilli counts were plated onto MRS and blood agar (Oxoid, UK). After 48h of incubation in a microaerobic environment (MRS plates), colonies were enumerated. The *Lactobacillus* spp. were identified according to Gram staining, colony and cell morphology, negative catalase reaction and carbohydrate fermentation patterns. Changes in lactobacilli counts in the ileum and colon were estimated by microbiological methods and PCR-DGGE. For histological analysis, tissue sections of the liver, spleen, kidney and lungs of the sacrificed mice were fixed in 10% of formaldehyde and embedded in paraffin. The samples were stained with haematoxylin and eosin, and by using van Gieson method. Alterative and inflammatory changes in the tissues were evaluated.

To determine the safety of six putative probiotic lactobacilli, a mixture of six *Lactobacillus* strains (*L. acidophilus* 821-3, *L. gasseri* E16B7, *L. gasseri* 177, *L. paracasei* 317, *L. paracasei* 1-4-2A and *L. fermentum* 338-1-1 (each strain in a concentration of 10^7 CFU per day) in drinking water was administered to 10 BALB/c mice (Scanbur BK AB, Sweden) for five consecutive days. Until cessation of the trial on day 15, the mice were fed with the commercial diet R-70 (Lactamin, Sweden). Five randomly selected mice were sacrificed on day 5, and the other five mice, on day 15. Samples for histological and microbiological analyses were collected.

Throughout both trials, the animals' activity, behaviour and general health were estimated daily. Namely, changes in the behaviour, coat texture, physical activity, and general health of animals but also changes in body weight, and

related changes in food and water consumption were observed according to OECD Guidance 2000 (OECD, 2000). All animal experiments were conducted under a protocol approved by the Ethics Committee on Animal Experiments of the Ministry of Agriculture of Estonia (protocol numbers 67/09.11.2006, 50/07.02.2006).

4.8. Clinical trials
(Papers II, IV, V, VI)

4.8.1. Ethics and study design

All trials' protocols were approved by the Ethics Committee of the University of Tartu. All trials were registered in Current Controlled Trials (ISRCTN 43435738, ISRCTN30946841, ISRCTN38739209 and ISRCTN45791894).

The present PhD study includes four clinical trials: a randomised double-blind placebo-controlled trial, two double-blind placebo controlled trials and an open-label trial. The expected primary outcomes of the clinical trials are presented in Table 3.

The randomisation list for the synbiotic trial (RDBPCCOT) was generated by a researcher who was not involved in recruitment or study visits and research (PhD student Piret Kõll). Sealed envelopes contained a study code number that corresponded to a particular treatment period (synbiotic or placebo period). The investigators and participants of the study were blinded to the randomisation code until all data were analysed. Volunteers were allocated to receive a synbiotic or a placebo for 3 weeks and after a 2-week washout period the volunteers were crossed over to another 3 weeks of placebo or synbiotic administration.

All participants in the DBPC probiotic trials received a probiotic cheese comprising *L. plantarum* TENSIA first for three weeks and the control cheese for another three weeks after a 2-week washout. The investigators and participants of study were blinded to the randomisation until all data were analysed.

In the open-label trial (OLT) for assessment of the safety, survival and persistence of putative probiotic strains, the participants consumed capsules containing lactobacilli for 5 days in two phases of the study (Table 4). Investigators and participants of the study were not blinded.

Table 3. Registration numbers and primary outcomes of performed clinical trials.

Study design **No of registration**	**Primary outcome**
RDBPCCOT (paper II) ISRCTN43435738	Effect of synbiotic intake on health indices, biochemical markers (including oxidative stress related markers) and faecal microflora.
OLT (paper IV) ISRCTN30946841	Safety of consumption of putative probiotic lactobacilli and detection of the best colonizer from among the five tested *Lactobacillus* strains in the two phases of trial.
DBPCT (paper V) ISRCTN38739209 acronym: TE1	Safety of consumption of probiotic cheese comprising *Lactobacillus plantarum* in healthy adults and survival of the probiotic strain in gastrointestinal tract and its effect on faecal lactoflora.
DBPCT (paper V) ISRCTN45791894 acronym: ELD	Safety of consumption of probiotic cheese comprising *Lactobacillus plantarum* in over 60-year-old subjects and survival of the probiotic strain in gastrointestinal tract and its effect on faecal lactoflora.

4.8.2. Study population

Study population involved persons who volunteered to participate, belonged to different age groups (adult and/or over 60 years) and considered themselves healthy. Participants were asked to maintain their normal diet but refrain from the consumption of probiotic products. All participants signed the written informed consent and were informed that they could withdraw from the study at any time.

Specific descriptions of all clinical trials are listed in Table 4.

4.8.3. Inclusion and exclusion criteria for trials

Inclusion criteria for selection of the subjects for all trials were: a desire to participate, appropriate age (over 18 years old), and without known health problems. Exclusion criteria included a history of gastrointestinal disease, food allergy and acute infection, cardiovascular diseases, use of any antimicrobial agent within the preceding month or use of any regular concomitant medication including non-steroidal anti-inflammatory drugs and antioxidant vitamins, pregnancy and breastfeeding. The withdrawal criteria from the trials included acute infections during the study, unwillingness to proceed with the study or relocation to new area.

4.8.4. Probiotic strains and prebiotic used in clinical trials

A list of the probiotic strains and the prebiotic used in the clinical trials is presented in Table 4. The doses of probiotic strains ranged from 1.5×10^8 to 5×10^{10} CFU, and were consumed daily in the probiotic period in the clinical trials. The probiotics were administered either as monocultures or mixed cultures.

In the randomised, double-blind, placebo-controlled crossover trial (RDBPCCOT) a mixture of probiotics (*L. fermentum* ME-3, *L. paracasei* 8700:2, *B. longum* 46) in capsules was applied. In addition, the participants consumed fructo-oligosaccharide (raftilose P95, Orafti, Belgium) in the above mentioned study. In the placebo period of the randomised, double-blind, placebo-controlled crossover trial (RDBPCCOT) the participants were administered maltodextrin.

The open-label trial (OLT) for assessment of the safety of putative probiotic strains was conducted. A mixture of 5 putative probiotic lactobacilli (*L. acidophilus* 821-3, *L. paracasei* 317, *L. fermentum* 338-1-1, *L. gasseri* 177, *L. gasseri* E16B7) in gelatine capsules at a daily dose of 5×10^{10} CFU was used in the first phase of the colonization study. In the second phase of the colonization study, the volunteers consumed *L. acidophilus* 821-3 as a monoculture (daily 1×10^{10} CFU).

The doses of *L. plantarum* TENSIA varied in the probiotic cheeses (26% fat content, semi-hard Edam-type) in both safety trials (DBPCT) (2×10^{10} CFU *vs* 1.5×10^8 CFU per 50 g cheese per day, respectively). In the placebo period of the double-blind, placebo-controlled trial (DBPCT) of both age groups the participants consumed control cheese (average 26% fat content, semi-hard Edam-type cheese without probiotic bacteria).

The viability of the encapsulated probiotics and putative probiotic lactobacilli, as well as of *L. plantarum* TENSIA in cheese was checked by cultivation before use in the human trials.

Table 4. Description of performed clinical trials.

Study design No of registration	No of participants	Age, range (median)	Probiotic	Dose per day, active component and placebo	Duration of treatment
RDBPCCOT (paper II)	n=53 F / M 41 / 12	20–60 (35.0)	*L. fermentum* ME-3 *L. paracasei* 8700:2 *B. longum* 46 Raftilose P95	3×10^9 CFU Raftilose P95 6.6 g Placebo – maltodextrin	3 weeks consumption of synbiotic / placebo 2 weeks wash-out 3 weeks consumption of placebo / synbiotic
OLT (paper IV)	Phase 1 n=9 F / M 5 / 5	22–40 (29.0)	*L. acidophilus* 821-3 *L. paracasei* 317 *L. fermentum* 338-1-1 *L. gasseri* 177 *L. gasseri* E16B7	5×10^{10} CFU capsulated bacteria	5 days consumption of putative probiotic strains 15 day wash-out period
	Phase 2 n=5 F / M 4 / 1	33–68 (41)	*L. acidophilus* 821-3	1×10^{10} CFU capsulated bacteria	5 days consumption of putative probiotic strains 15 day wash-out period
DBPCT (paper V) acronym: TE1	n=12 F / M 7 / 5	21–43 (27.0)	*L. plantarum* TENSIA comprising cheese	50 g of cheese 2×10^{10} CFU	3 weeks consumption of probiotic cheese 2 weeks wash-out 3 weeks consumption of placebo cheese
DBPCT (paper V) acronym: ELD	n=18 F / M 17 / 1	61–81 (69.5)	*L. plantarum* TENSIA comprising cheese	50 g of probiotic or regular cheese 1.5×10^8 CFU	3 weeks consumption of probiotic cheese 2 weeks wash-out 3 weeks consumption of placebo cheese

DBPCT, double-blind, placebo-controlled trial; RDBPCCOT, randomised, double-blind, placebo-controlled crossover trial; OLT, open-label trial, F, female, M, male.

4.8.5. Data collection in clinical trials

Anthropometric indices such as weight and height were measured. Body mass index (BMI) was calculated as the weight (kg) divided by height squared (m^2) (WHO, 1998). Clinical parameters included blood pressure measurements and blood sampling.

The volunteers of the trials were daily or once a week questioned about their general welfare, intestinal function (flatulence, bloating, stool frequency, abdominal pain) and adverse effects.

The blood samples from the antecubital vein, faecal and urine samples were collected before and at the end of all clinical trials.

The following blood indices were measured: inflammatory markers (white blood cells (WBC), high sensitive C-reactive protein (hs-CRP)), haematological indices (haemoglobin, red blood cells, and platelets) and liver and kidney functional indices (aspartate aminotransaminase (ASAT), alanine aminotransaminase (ALAT), albumin and serum creatinine), metabolic indices (blood glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides). The standard laboratory methods were applied using certified assays in the local clinical laboratory.

For measurement of oxidative stress-related parameters like total antioxidative activity (TAA), total antioxidative status (TAS), ratio of oxidized to reduced glutathione (GSSG/GSH), baseline diene conjugates of low-density lipoproteins (BDC-LDL), and the oxidized LDL level (oxLDL) of the blood sera were used. Testing of oxidative stress related indices was performed in cowork with the researchers of the Department of Biochemistry of University of Tartu.

To estimate the survival of the consumed putative probiotics and their persistence after cessation of consumption up to 15–20 days in GI tract the faecal samples were used. The prevalence and counts of the consumed strains were assessed by different cultural and molecular methods (arbitrarily primed polymerase chain reaction (AP-PCR), PCR-denaturing gradient gel electrophoresis (PCR-DGGE), real-time PCR) were applied. The real-time PCR was performed in Karolinska Institute in Sweden.

The survival and persistence of *L. plantarum* TENSIA was evaluated using the above mentioned methods, exept PCR-DGGE.

4.8.5.1. Measurement of oxidative stress related indices in synbiotic trial

Blood samples were collected from the antecubital vein before and after treatment of probiotics. Serum was analysed for total antioxidative activity (TAA), total antioxidative status (TAS) and ratio of oxidized to reduced glutathione (GSSG/GSH) (present study).

The following oxidative stress related markers, such as blood TAA, TAS, GSSG/GSH (paper II), baseline diene conjugates of low-density lipoproteins (BDC-LDL) and the oxidized LDL level (oxLDL) were measured (present study, Mikelsaar *et al.,* 2008).

TAA of serum was assessed by the linolenic acid test (LA-test) as described previously by Kullisaar *et al.* (2002). This test evaluates the ability of the blood sera to inhibit lipid peroxidation.

TAS of serum was measured with commercially available kit (TAS, Randox Laboratories Ltd. Ardmore, UK) as described elsewhere (Kullisaar *et al.*, 2003), water-soluble vitamin E (Trolox) serving as standard. This method is based on the inhibition of the absorbance of the ferrylmyoglobin radicals of 2,2'-azinobis-ethylbenzothiazoline 6-sulfonate (ABTS+) generated by activation of metmyoglobin peroxidase with H_2O_2 and indicates the antioxidativity of the blood sera in water fractions.

Total glutathione and oxidized glutathione were measured by using the method of Griffith (1980) as described elsewhere (Kullisaar *et al.*, 2003). The glutathione content was calculated on the basis of a standard curve generated with known concentration of glutathione. Amount of GSH was calculated as a difference between the total glutathione and GSSG (total glutathione – GSSG = GSH). The glutathione content was expressed as the ratio of oxidized to reduced glutathione (GSSG/GSH).

The levels of the oxidized low-density lipoprotein (oxLDL) were measured using an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden).

Baseline diene conjugates of LDL were measured by determining the level of LDL diene conjugates using a method that has been validated and reported in detail (Ahotupa *et al.*, 1998). In brief, serum LDL was isolated by precipitation with buffered heparin citrate. The amount of peroxidized lipids in the samples was determined by the degree of conjugated diene double bonds. Lipids were extracted from the samples by a mixture of chloroform and methanol (2:1), dried under nitrogen, redissolved in cyclohexane, and analysed spectrophotometrically at 234 nm. For BDC-LDL, the coefficient of variance for within-assay and between-assay precision was 4.4% and 4.5%, respectively.

4.8.6. Detection of *Helicobacter pylori* in faeces by a stool kit (Papers II)

The presence of *H. pylori* was tested in 53 healthy adult volunteers without gastric symptoms who participated in the randomised, double-blind, placebo-controlled crossover trial.

H. pylori colonization was tested from faecal samples at the beginning of the study as a baseline value and at the end of the study applying the HpSA-test (ImmunoCard STAT HpSA, Meridian Bioscience Europe, Milan, Italy)

(Andreson *et al.*, 2003; Krogfelt *et al.*, 2005). According to the results of the HpSA-test the subjects were divided into *H. pylori*-positive and *H. pylori*-negative study group.

4.9. Statistical analysis

Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael CA, USA) and R 2.6.2 (A Language and Environment, http://www.r-project.org). The following tests were employed: Fisher exact test, Student's t-test or Mann-Whitney rank sum test (comparison of antagonistic activity of probiotic), paired t-test or Wilcoxon signed rank test (comparisons of biochemical indices in human trials), Spearman rank order correlation (comparisons between antimicrobial activity and the production of organic acids). Data of antioxidative and antagonistic activity of probiotics were analysed using a Student's t-test or Mann-Whitney rank sum test with the Bonferroni correction for multiple groups.

The differences were considered significant when p value was < 0.05.

5. RESULTS AND DISCUSSION

5.1. Functional properties of probiotics and putative probiotics

5.1.1. Screening for colonizing potential (auto-aggregation ability, acid, bile and pancreatin tolerance) and haemolytic activity of putative probiotics

We tested auto-aggregation ability, and acid, bile and pancreatin tolerance as indicators of a simulated GI environment and haemolytic activity of lactobacilli strains. The strains were scored according to the results of auto-aggregation and acid, bile, and pancreatin tolerance (Table 5).

All tested putative probiotic strains showed similar bile and pancreatin tolerance to tested concentrations, while differences in acid tolerance and auto-aggregation were detected.

The main differences between *Lactobacillus* strains were found in score of auto-aggregation and survival at low pH. The highest score was shown by *L. acidophilus* 821–3 and *L. gasseri* E16B7. According to the scoring results, the sum of *L. acidophilus* 821–3 showed the highest and *L. plantarum* TENSIA the lowest score (Table 5). Similarly to our study, differences in the acid tolerance but also in the bile tolerance of intestinal *Lactobacillus* strains have been demonstrated elsewhere (Delgado *et al.*, 2007).

The ability to survive in and to adhere to the GI tract is the presumed desirable characteristics required for probiotic activity of lactobacilli (Morelli, 2000). Thus, selected bile- and acid-tolerant and good adherent strains manifest good transit tolerance; hence, they may be able to survive in the harsh environmental conditions of the human GI tract (FAO/WHO, 2002).

Detection of the haemolytic potential of putative probiotics is required for safety reasons (FAO/WHO, 2002). In our study putative probitic strains were tested for haemolytic activity using either human or horse blood. None of the tested lactobacilli caused the lysis of erythrocytes on human and horse blood agar while complete lysis (β-haemolysis) was registered in case of positive controls (*Streptococcus pyogenes* and *Staphylococcus aureus*). This finding is in accordance with a previous study showing non-haemolytic activity among dairy and human origin lactobacilli (Maragkoudakis *et al.*, 2009).

Table 5. Auto-aggregative properties and acid, bile and pancreatin tolerance of six putative probiotic *Lactobacillus* strains and of the probiotic *L. plantarum* TENSIA.

Putative probiotic strains	Species	Auto-aggregation[a]	Survival at			
			pH [b]	Oxgall (% w/v)[c]	Pancreatin (% w/v)[d]	Score
821-3	*L. acidophilus*	2	2	2	2	8
E16B7	*L. gasseri*	2	1	2	2	7
177	*L. gasseri*	2	0	2	2	6
317	*L. paracasei*	1	1	2	2	6
338-1-1	*L. fermentum*	1	1	2	2	6
1-4-2A	*L. paracasei*	1	1	2	2	6
TENSIA	*L. plantarum*	0	1	2	2	5

[a]Auto-aggregation: score 0, no aggregation; score 1, intermediate aggregation; and score 2, strong aggregation; [b]Acid tolerance: score 0, survival only at pH > 3.0; score 1, survival at pH 3.0; and score 2, survival at pH ≤ 2.5; [c]Bile tolerance: score 0, no survival at oxgall concentration 2% (w/v); score 2, survival at oxgall concentration 2%; [d]Pancreatin tolerance: score 0, no survival at pancreatin concentration 0.5% (w/v); score 2, survival at pancreatin concentration 0.5%.

5.1.2. Antibiotic susceptibility pattern of putative probiotics

In this study, all tested *Lactobacillus* strains were susceptible to ampicillin, gentamicin, erythromycin, tetracycline, chloramphenicol and rifampicin. This shows that the tested strains are phenotypically susceptible to antibiotics belonging to the EFSA list for mandatory testing. *L. fermentum* 338-1-1, *L. paracasei* 317 and *L. paracasei* 1-4-2A were resistant to vancomycin. All tested strains were resistant to ciprofloxacin except *L. paracasei* 1-4-2A and *L. fermentum* 338-1-1. Trimethoprim-resistant strains were *L. gasseri* E16B7, *L. gasseri* 177, *L. paracasei* 317 and *L. paracasei* 1-4-2A (Table 6).

In general, there are differences in the antibiotic susceptibility pattern of lactobacilli at the species and fermentation type levels (Mändar *et al.*, 2001). Different species of lactobacilli have high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen and Wind, 2003; Mikelsaar, 2011). However, natural resistance to vancomycin, which is not transferable, is characteristic to FHEL and OHEL groups of lactobacilli (Mändar *et al.*, 2001; Delgado *et al.*, 2007). This intrinsic resistance to vancomycin is based on the presence of D-Ala-D-lactate in their peptidoglycan rather than natural D-Ala-D-Ala dipeptide (Klein *et al.*, 2000).

In our study, all *Lactobacillus* strains tested did not express resistance to erythromycin or tetracycline. This finding suggests the absence of acquired resistance among these intestinal isolates.

Table 6. Antibiotic susceptibility of six putative probiotic *Lactobacillus* strains and of the probiotic *L. plantarum* TENSIA.

Lactobacilli		**Antibiotic[a] with MIC (μg/ml) as follows:**								
Strain	**Species**	**AM**	**VA**	**GM**	**EM**	**TC**	**CL**	**CI**	**RI**	**TS**
821-3	*L. acidophilus*	0.023	0.25	2	0.25	1	2	>32	0.38	0.75
E16B7	*L. gasseri*	0.19	1	4	0.19	3	2	>32	0.047	>32
177	*L. gasseri*	0.031	0.75	2	0.047	0.5	0.75	>32	0.064	>32
317	*L. paracasei*	1	≥256	1.5	0.38	0.75	3	>32	0.125	>32
1-4-2A	*L. paracasei*	0.064	≥256	0.75	0.25	0.75	3	1	0.094	>32
TENSIA	*L. plantarum*	0.25	nd	1.5	0.19	8	2	>32	nd	nd
338-1-1	*L. fermentum*	0.125	≥256	0.5	0.125	2	2	2	0.19	0.5
Gram-positive bacteria *		16	32	16	8	16	32	4	4	4/76
MIC breakpoint values for *Lactobacillus* spp (μg/ml)**		2	4	1	4	16	16	4	32	32
Fermentation groups***										
OHOL		1	2	16	1	4	4	n.r.	n.r.	n.r.
OHEL		2	n.r.	16	1	8	4	n.r.	n.r.	n.r.
FHEL		4	n.r.	16	1	8	4	n.r.	n.r.	n.r.

[a]AM, ampicillin; VA, vancomycin; GM, gentamicin; EM, erythromycin; TC, tetracycline; CL, chloramphenicol; CI, ciprofloxacin; RI, rifampicin; TS, trimethoprim-sulfamethoxazole; nd, not detected; n.r. not required; OHOL, obligately homofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli. 821-3, E16B7 and 177 belong to the OHOL group; 317, 1-4-2A and TENSIA belong to the FHEL group and 338-1-1 belongs to the OHEL group. Recommendations for MIC interpretation: at the gram-positive microorganism level (* Jorgensen and Turnidge 2003), lactobacilli at the genus level (** EC 2002) and at the fermentation group of lactobacilli level (*** EFSA, 2008).

However, some studies reported the evidence of resistance to erythromycin and/or tetracycline among human faecal *Lactobacillus* isolates (Delgado *et al.*, 2007). These isolates may harbour acquired resistance genes identified encoded resistance to tetracycline (*tet* (M), *tet* (W), *tet* (O) and *tet* (O/W)), erythromycin and clindamycin (*erm* (B)) (Ammor *et al.*, 2007; Ammor *et al.*, 2008).

Lactobacillus strains carrying acquired resistance to antimicrobial(s) should not be used as probiotics and should therefore be discarded from further testing for selection of candidate probiotics (EFSA, 2008).

5.1.3. Antagonistic activity of probiotics

We aimed to find out the antagonistic activity of commercial probiotic lactobacilli against facultatively anaerobic gram-negative target bacteria: uropathogenic *E. coli, Salmonella enterica* ssp. *enterica* and *Shigella sonnei* both in microaerobic and in anaerobic conditions using different media and taking account of the pathogens' niche in the GI tract. The antagonistic activity of bifidobacteria (strict anaerobes) was tested only in anaerobic conditions.

Differences in the antagonistic activity of probiotic strains depending on growth environments were found. Namely, *L. rhamnosus* GG and *L. plantarum* 299v demonstrated lower inhibition in anaerobic conditions on solid media compared to *L. paracasei* 8700:2 that belongs to the same fermentation group (FHEL). The inhibitory activity of only *L. paracasei* 8700:2 was not dependent on either growth conditions (microaerobic *vs* anaerobic environment) or growth medium (solid *vs* liquid media) (Table 7). The antagonistic activity of *L. acidophilus* La5 (OHOL) was the weakest in microaerobic conditions, while the increase in anaerobic conditions was significant. Similarly, the weakest antimicrobial activity of the OHOL group, which increases in anaerobic conditions, has been shown in an earlier study (Annuk *et al.*, 2003).

In contrast to our findings, Annuk *et al* (2003) reported that the antagonistic activity of the OHEL group decreased in an anaerobic milieu. We found that the antagonistic activity of *L. fermentum* ME-3 (OHEL group) increased in anaerobic mileu, and *L. fermentum* ME-3 was the most active probiotic *Lactobacillus* strain in anaerobic conditions on a solid medium (Table 7).

L. plantarum 299v (FHEL group) demonstrated the highest antagonistic activity under microaerobic conditions but its inhibitory effect decreased significantly under anaerobic conditions. Similarly, *L. plantarum* TENSIA showed also high antagonistic activity in a microaerobic environment. In contrast to this finding, Annuk and co-workers have shown that the OHEL group has higher inhibitory activity compared to the FHEL group (Annuk *et al.*, 2003). Thus, our study showed that the antagonistic activity of the probiotic is more strain-specific than fermentation type specific.

Bifidobacteria (*B. lactis* Bb12 and *B. longum* 46) did not suppress 4 facultatively anaerobic gram-negative target bacteria on solid agar but their inhibitory effect was detectable using a liquid medium. There was no significant

difference in inhibitory activity (pathogens inhibition values were in the range 3.2 up to 6.1 $\log_{10}$ CFU/ml) between the *Bifidobacterium* strains.

Table 7. Antagonistic activity of the commercial probiotic lactobacilli and of the probiotic *L. plantarum* TENSIA grown in microaerobic and anaerobic environments against gram-negative (two uropathogenic *E. coli* strains, *Salmonella enterica* ssp. *enterica* and *Shigella sonnei*) tested on modified MRS agar and liquid media (expressed as mean ± SD).

Group	Strain	Solid medium (inhibition zone values, mm)			Liquid medium (inhibition, $\log_{10}$ CFU)		
		Microaerobic	Anaerobic	p value	Microaerobic	Anaerobic	p value
OHEL	ME3	22.8 ± 4.6	23.6 ± 2.0	<0.001	5.1 ± 1.9	5.6 ± 1.4	0.626
FHEL	GG	22.9 ± 3.5	19.3 ± 1.5	<0.001	4.4 ± 2.0	5.3 ± 1.8	0.213
	299v	26.7 ± 3.1	21.8 ± 1.1	<0.001	5.5 ± 1.1	3.0 ± 2.4	0.007
	TENSIA	25.2 ± 0.1	nd	nd	nd	nd	nd
	8700:2	18.8 ± 2.8	18.4 ± 2.0	0.891	6.3 ± 0.7	5.4 ± 1.7	0.326
OHOL	La5	2.7 ± 2.4	15.5 ± 1.2	<0.001	1.2 ± 1.0	4.0 ± 2.0	0.002

SD, standard deviation; nd, not determined
p values, difference between the microaerobic and the anaerobic environment
ME-3, *L. fermentum* ME-3; GG, *L. rhamnosus* GG; 299v, *L. plantarum* 299v; 8700:2, *L. paracasei* 8700:2; La5, *L. acidophilus* La5; TENSIA, *L. plantarum* TENSIA
OHOL, obligately homofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli

Selected assays for ranking probiotics against target pathogens

Further, we applied ranking of probiotics according to their antagonistic ability against facultatively anaerobic gram-negative pathogens in liquid media that resemble the gastrointestinal environment better than solid media. Different probiotics were ranked as expressing high, moderate and low activity in different atmospheric conditions mimicking the milieu in the GI tract (Table 8).

The pyelonephritic strain of *E. coli* (ATCC 700336) was highly suppressed by *L. rhamnosus* GG and both strains of bifidobacteria, but no significant activity was found against cystitic *E. coli* (ATCC 700414). Effective probiotics against *S. enterica* ssp. *enterica* were *L. paracasei* 8700:2, *L. plantarum* 299v and *L. fermentum* ME-3 showing high activity in a microaerobic milieu. *L. fermentum* ME-3 and both bifidobacteria expressed high activity against *S. sonnei* in an anaerobic milieu.

Antagonistic activity against *H. pylori* was evaluated only on solid media. The highest antagonistic activity (Figure 3) was expressed by *B. longum* 46 ($p<0.05$) anaerobically. However, under microaerobic conditions characteristic of the stomach, high antagonistic activity was expressed by lactobacilli belonging to the FHEL group (*L. paracasei* 8700:2, *L. rhamnosus* GG and *L. plantarum* 299v).

Table 8. Ranking of probiotic strains according to their antagonistic activity against target pathogens in the microaerobic and in the anaerobic environments.

Target bacteria	Environment of action in liquid media	OHEL		FHEL		OHOL	Bifidobacteria	
		ME-3	GG	299v	8700:2	La5	Bb12	B46
E. coli ATCC 700336	Anaerobic	M	H	L	M	M	H	H
E. coli ATCC 700414	Anaerobic	M	M	L	M	M	M	M
Salmonella enterica spp *enterica* ATCC 13076	Microaerobic	H	M	H	H	L	nd	nd
	Anaerobic	M	M	L	M	M	L	L
Shigella sonnei ATCC 25931	Anaerobic	H	M	L	M	M	H	H

nd, not determined

Different probiotics were ranked as expressing high (H), moderate (M) and low (L) activity in different atmospheric conditions mimicking the milieu in GI tract.

ME-3, *L. fermentum* ME-3; GG, *L. rhamnosus* GG; 299v, *L. plantarum* 299v; 8700:2, *L. paracasei* 8700:2; La5, *L. acidophilus* La5

Bb12, *B. lactis* Bb12, B46, *B. longum* 46

K. Lõivukene showed that the fermentation pattern of lactobacilli did not correlate with their antagonistic activity against *H. pylori* (Lõivukene, 2003). In contrast to that study, we found a significant difference in the antagonistic activity of lactobacilli according to their fermentation pattern. The FHEL group expressed higher antagonistic activity towards *H. pylori* compared to the other fermentation groups.

The inhibitory activity of probiotic bacteria against the strictly anaerobic *C. difficile* reference strain was low (6–8 mm), expressed by both bifidobacteria strains and *L. paracasei* 8700:2.

Further research will be needed to confirm if the differences *in vitro* experiments of the antimicrobial activity of probiotic strains in different environments of GI tract biotypes are in accordance with corresponding animal studies. This could precede clinical efficacy studies of adjunct treatment with probiotics in cure of different gastrointestinal and urinary tract infections.

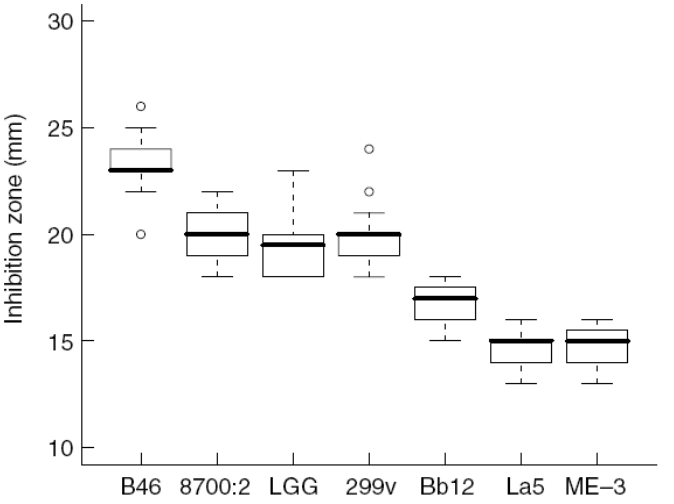


Figure 3. Antagonistic activity of probiotic bacteria against the *H. pylori* reference strain (NCTC 11637) on solid media under microaerobic conditions. Data present median inhibition (–) and distribution. Boxes display 25th –75th quartile area, bars display 10th–90th percentage area. La5, *L. acidophilus* La5, LGG, *L. rhamnosus* GG, 8700:2, *L. paracasei* 8700:2; 299v, *L. plantarum* 299v; ME-3, *L. fermentum* ME-3; B46, *B. longum* 46; Bb12, *B. lactis* Bb12.

B46 > 8700:2 (p=0.001), B46 > ME-3 (p=0.001), 8700:2 > ME-3 (p=0.001)

Association between inhibitory activity of lactobacilli and production of organic acids and pH

We did not find significant differences between the production of lactic, acetic or succinic acids of the tested probiotic lactobacilli strains either under anaerobic or microaerobic conditions (data not shown). However, all tested lactobacilli produced to some extent more lactic acids under microaerobic than under anaerobic conditions. A higher amount of ethanol was produced by *L. fermentum* ME-3 in the anaerobic environment than in the microaerobic environment (96.3 mmol/l *vs* 54.9 mmol/l). No ethanol was produced by *L. rhamnosus* GG, *L. plantarum* 299v or *L. acidophilus* La5.

Annuk *et al.* (2003) demonstrated differences in the production of organic acids between the fermentation groups of lactobacilli. Namely, the FHEL group produced abundant lactic acid and the OHEL group produced acetic acid.

We found a positive correlation between production of lactic acid and inhibitory activity of lactobacilli after cultivation in microaerobic conditions ($r=0.457$; $p=0.043$, $n=20$) and inhibitory activity of bifidobacteria after cultivation in anaerobic conditions ($r=0.862$, $p=0.005$, $n=8$). The amount of acetic acid and the inhibitory activity of lactobacilli and bifidobacteria cultured in anaerobic conditions were negatively correlated ($r=–0.428$, $p=0.006$; $r=–0.862$, $p=0.006$, respectively).

Following microaerobic cultivation, pH values in liquid media correlated inversely with antagonistic activity of five probiotic lactobacilli ($r=–0.530$, $p=0.016$).

A previous study has revealed correlations between antagonistic activity and production of organic acids and pH (Annuk *et al.*, 2003). The findings of our study for selection of probiotics or putative probiotics are in concordance with the results of the aforementioned study of Annuk and coauthors.

5.1.4. Total antioxidative activity of probiotics strains

In our study the detected indices, *i.e.* TAA and TAS, include both enzymatic and non-enzymatic antioxidative compounds of lactic acid bacteria. The probiotic strain *L. fermentum* ME-3 expressed the highest values of anti-oxidative activity compared to the other tested probiotic strains (Table 9).

Annuk *et al*. (2003) showed that the intestinal lactobacilli belonging to the OHOL group expressed the highest TAA values although some particular strains from the other groups such as FHEL and OHEL also showed high values of TAA.

Table 9. Antioxidative activity of probiotic lactic acid bacteria (mean ± SD).

Probiotic strains	TAA (%)	TAS (mmol/l)
L. acidophilus La5	16 ± 4	0.08 ± 0.06
L. rhamnosus GG	16 ± 7	0.09 ± 0.03
L. plantarum 299v	12 ± 5	0.01 ± 0.02
L. paracasei 8700:2	15 ± 4	0.03 ± 0.03
L. fermentum ME-3	24 ± 4	0.18 ± 0.05
B. lactis Bb12	11 ± 6	0.03 ± 0.03
B. longum 46	11 ± 4	0.10 ± 0.08

TAA, total antioxidative activity; TAS, total antioxidative status; SD, standard deviation.
The total antioxidative values of probiotic bacteria were considered high if TAA was >20% and TAS was > 0.1 mmol/l.
All TAA and TAS values of the tested probiotic bacteria *versus L. fermentum* ME-3 were significantly lower ($p<0.05$)

In summary, the functional properties of the tested commercial probiotics are variable and strain specific. We found that the uropathogenic strain of *E. coli* was highly suppressed by *L. rhamnosus* GG and both strains of bifidobacteria. *L. fermentum* ME-3 and both bifidobacteria expressed high activity against *S. sonnei*. The effective probiotics against *S. enterica* ssp. *enterica* were *L. paracasei* 8700:2, *L. plantarum* 299v and *L. fermentum* ME-3.

B. longum 46, *L. paracasei* 8700:2, *L. rhamnosus* GG and *L. plantarum* 299v strains showed moderate antagonistic activity against *H. pylori* under microaerobic conditions on solid media.

For a further synbiotic trial, three commercial strains (*B. longum* 46, *L. paracasei* 8700:2 and *L. fermentum* ME-3) were selected according to the results of *in vitro* tests. The probiotic strain *L. fermentum* ME-3 showed the highest antioxidative activity. *B. longum* 46 expressed the highest antagonistic activity against *H. pylori*. *L. paracasei* 8700:2 was the only strain whose antimicrobial activity did not differ in different growth conditions (microaerobic *vs* anaerobic environment) or in different growth media (solid *vs* liquid media).

5.2. *In vivo* animal trials

Two different mice lines, BALB/c and NIH, were used to determine the safety of six putative probiotic lactobacilli (*L. acidophilus* 821-3, *L. gasseri* E16B7, *L. gasseri* 177, *L. paracasei* 317, *L. paracasei* 1-4-2A and *L. fermentum* 338-1-1) and of the probiotic *L. plantarum* TENSIA, respectively. The daily doses (10^9 CFU of probiotic strains) used were concordant with data of literature (Pavan *et al.*, 2003).

Oral administration of these lactobacilli did not cause any change in the body weight, intestinal inflammation, adverse behavioral effect and general health status of the treated mice. The heart blood, liver, kidney and lung samples obtained at autopsy were sterile in all mice treated with lactobacilli.

The spleen culture of one BALB/c mouse (mouse H5, sacrificed on day 5) was positive for lactobacilli both on MRS and blood agar media. Two different species were identified by API – *L. paracasei* subsp. *paracasei* and *L. plantarum*. AP-PCR typing revealed that the banding pattern of *L. plantarum* was not similar to the fingerprint patterns of any of the *Lactobacillus* strains that were administered to the mice but the banding pattern of *L. paracasei* subsp. *paracasei* was identical to that of the *L. paracasei* strain 1-4-2A.

Mouse H5 with a positive spleen culture showed no pathological changes in any of the organs, including the kidney and lungs. The translocated strain *L. paracasei* 1-4-2A was excluded from further study (human trial) as a non-safe strain for human consumption.

5.3. Clinical trials

5.3.1. Selection of LAB strains for human trials

Four intervention studies were carried out using monostrain or multispecies bacteria in capsules or probiotic cheese comprised *L. plantarum* TENSIA.

The bacteria used in these clinical trials were selected according to their favourable functional properties and colonizing potential, and earlier confirmed safety in animal models (Table 10). The lactobacilli and the bifidobacterium, which belonged to the mixture, had no inhibitory effect on each other.

Table 10. Favourable colonizing potential and functional properties of the strains serving as the basis for selection of the following clinical trials.

Strains	Favourable functional properties
L. fermentum ME-3	high antioxidative activity, antagonistic activity
L. paracasei 8700:2	antagonistic activity
B. longum 46	antagonistic activity
L. plantarum TENSIA	good viability in simulated GI tract conditions confirmed safety in an animal model high antagonistic activity
L. acidophilus 821-3 *L. paracasei* 317 *L. fermentum* 338-1-1 *L. gasseri* 177 *L. gasseri* E16B7	All strains expressed: a) good viability in simulated GI tract conditions b) confirmed safety in an animal model

5.3.2. Performed clinical trials

Our study included one randomised, double-blind, placebo-controlled crossover trial, one open-label trial and two double-blind, placebo-controlled trials.

The crossover clinical trials were chosen for comparison within an individual, as each individual is its own control. As there is usually less variability within a subject than between different subjects, the precision of observation will increase. A two-week washout period was considered long enough to wash out the ingested probiotics because commonly probiotic persists in the GI tract less than two weeks (Goldin *et al.*, 1992; Saxelin *et al.*, 1995; Jacobsen *et al.*, 1999; Mätto *et al.*, 2006).

The study population of the probiotic cheese studies represented generally healthy adults and healthy adults aged over 60 years.

Health assessment was based on participant interviews conducted prior to the study and on self-reported questionnaires for monitoring the symptoms and adverse effects, different blood cellular and biochemical indices and several measurements, such as measurements of body weight or blood pressure.

The main purpose of the clinical trials and the drop-outs are shown in Table 11.

5.3.3. Synbiotic trial

Substantiation of the efficacy of the synbiotic was performed with a controlled clinical trial. In our study we tested the efficacy of a probiotic multispecies combination (*L. fermentum* ME-3, *L. paracasei* 8700:2, *B. longum* 46) with added prebiotic raftilose P95 on oxidative stress related markers of healthy adults.

Healthy volunteers were allocated to receive synbiotic (group 1) or placebo (group 2) for 3 weeks. After 2-week washout period the volunteers were crossed over to another 3 weeks of placebo or synbiotic administration. Except for the fact that more women than men participated in the study (77% *vs* 23%), the subjects were well balanced over the two study groups with respect to baseline characteristics such as age, BMI, blood pressure, biochemical indices (glucose, lipids) and presence of *H. pylori* (Table 12).

There were no significant changes in BMI, blood pressure and blood biochemical indices during the synbiotic trial (data not shown).

Table 11. The purpose of clinical trials and reasons for drop-out.

Study	No of participants completed the study	Main purpose of trial	Strains used	Drop-outs in clinical trials
RDBPCCOT (paper II)	n=53	Evaluation of efficacy (antioxidative markers)	*L. fermentum* ME-3 *L. paracasei* 8700:2 *B. longum* 46	n=8: non-compliance (n=1), pregnancy (n=2), acute infection and antimicrobial treatment (n=2), diarrhea (n=1), rheumatic fever (n=1), heart arrhythmia (n=1)
DBPCT (paper V)		Assessment of survival/persistence and safety of *L. plantarum* TENSIA	*L. plantarum* TENSIA	
TE1	n=12	2 studies		n=1: non-compliance
ELD	n=18			n=3: elevated blood pressure
OLT (paper IV)	n=14	Assessment of survival/persistence and safety of the selected 5 strains in human trial	*L. acidophilus* 821-3 *L. paracasei* 317 *L. fermentum* 338-1-1 *L. gasseri* 177 *L. gasseri* E16B7	n=1: acute infection and antimicrobial treatment
	n=5	Assessment of survival/persistence and safety of a selected strain in human trial	*L. acidophilus* 821-3	No drop-outs

RDBPCCOT, randomised, double-blind, placebo-controlled crossover trial; DBPCT, double-blind, placebo-controlled trial; OLT, open-label trial

Table 12. Baseline characteristics of the synbiotic study groups (expressed as mean ± SD).

	Total group (n=53)	Group 1 (n=27)	Group 2 (n=26)
Sex (n)			
Female	41	20	21
Male	12	7	5
Age (years)	36.3 ± 11.8	34.9 ± 11.7	37.8 ± 12.0
BMI (kg/m^2)	24.7 ± 4.8	25.4 ± 5.6	24.0 ± 3.8
Blood pressure (mmHg)			
Systolic	122.8 ± 15.5	122.7 ± 15.6	122.8 ± 15.7
Diastolic	80.3 ± 12.2	80.4 ± 12.4	80.2 ± 12.2
Glucose, mmol/l	4.8 ± 0.6	4.7 ± 0.5	4.8 ± 0.6
Total cholesterol, mmol/	5.2 ± 1.1	5.0 ± 0.9	5.5 ± 1.3
HDL, mmol/l	1.8 ± 0.3	1.7 ± 0.4	1.9 ± 0.3
LDL, mmol/l	3.4 ± 1.0	3.2 ± 0.9	3.5 ± 1.2
Triglycerides, mmol/l	1.2 ± 0.6	1.1 ± 0.5	1.2 ± 0.6
Presence of *H. pylori* (yes/no)	28 / 25	13 / 14	15 / 11

5.3.3.1. Improvement in oxidative stress related markers due to synbiotic intervention

For detection of oxidative stress related markers, modified, mainly oxidized, forms of LDL were tested in blood sera. Oxidized LDL (oxLDL) is an important marker of oxidative stress while baseline diene conjugates of LDL (BDC–LDL) is a specific indicator of circulating mildly oxidized LDL.

The BDC-LDL values decreased significantly in the blood of volunteers at the end of the synbiotic treatment period compared to baseline values (mean 15.2 *vs* 12.7 μM/l, $p<0.001$). A significant reduction was also seen at the end of the synbiotic period as compared to the end of the placebo period (mean 12.7 *vs* 14.6 μM/l, $p=0.035$) (Figure 4 a).

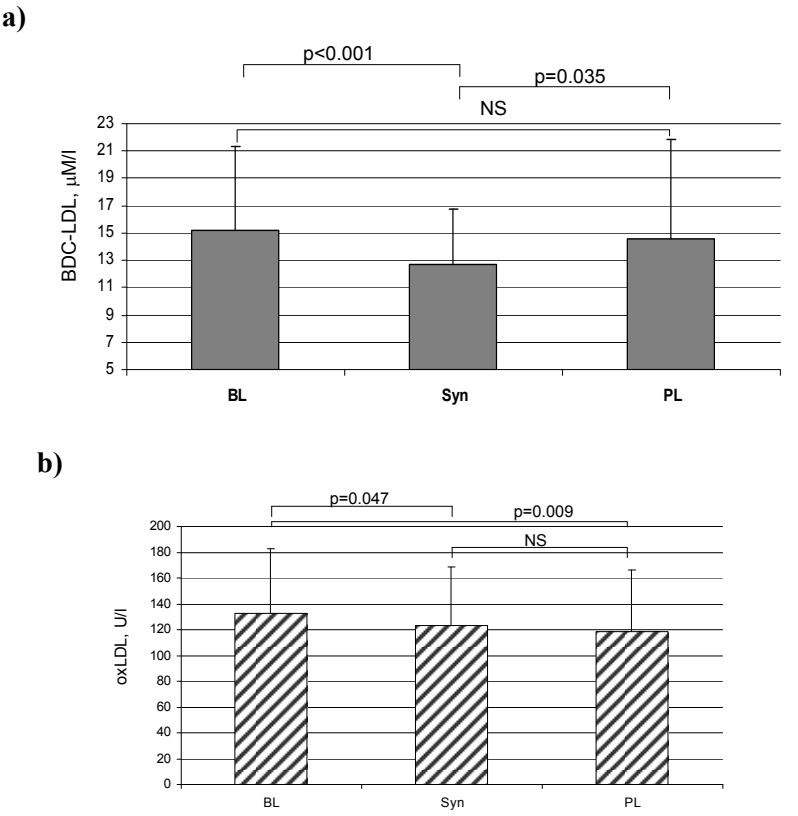


Figure 4. Changes in the level of BDC-LDL (a) and in oxLDL (b) values during the synbiotic trial.

BL, baseline; Syn, end of synbiotic consumption; PL, end of placebo period. NS, not significant

After the 3-week consumption of the synbiotic product we detected also a reduction of oxLDL values, however, similar changes were also found in the placebo period (Figure 4 b). The decrease of the oxLDL values in the placebo period may be caused by the unexpected carryover effect on synbiotic consumption in the crossover study. There were no significant differences in the oxLDL values at the end of the synbiotic period and at the end of the placebo period.

Clinical studies have shown that BDC-LDL is closely related to atherosclerosis and known atherosclerosis risk factors. As an indicator of the risk of the disease, BDC-LDL clearly exceeds the sensitivity and specificity of common markers, successfully revealing mild oxidation of LDL (Ahotupa and Asankari, 1999; Brizzi *et al.,* 2004). The BDC-LDL method distinctly improves possibilities for the diagnosis, follow-up of treatment, and basic research of cardiovascular diseases.

The revealed protection of LDL molecules from oxidative damage is evidently due to the high antioxidative properties of *L. fermentum* ME-3 from the probiotic mix. *L. fermentum* ME-3 is capable of alleviating oxidative stress- and inflammation-related damages in the intestinal cells (Truusalu *et al.*, 2004). High antioxidative activity has also been detected in probiotic cheese with ME-3 (Songisepp *et al.*, 2004). Moreover, the consumption of the probiotic *L. fermentum* ME-3 in fermented goat milk and in capsules increased the total antioxidative status (TAS) in healthy subjects (Kullisaar *et al.*, 2003; Songisepp *et al.*, 2005).

Improvement in blood antioxidative activity in H. pylori*-positive subjects*

In our synbiotic study healthy volunteers (n=53) without gastric symptoms were divided into two groups according to the presence of *H. pylori* in the stool sample. 28 persons were colonized with *H. pylori* (53%) and 25 were *H. pylori*-negative. *H. pylori*-positive individuals were significantly older than *H. pylori*-negative ones (39.9 ± 11.2 *vs* 32.3 ± 11.4 years, p=0.018). There was no difference in body mass index between the *H. pylori*-positive and *H. pylori*-negative subjects (BMI 25.6 ± 5.0 *vs* 23.8 ± 4.5, p>0.05).

We evaluated the impact of consumption of a synbiotic product on the antioxidative activity markers of blood (TAS, GSSG/GSH) in asymptomatic *H. pylori*-colonized persons. A positive change in the antioxidative markers was the reduction of the ratio of oxidized to reduced glutathione (mean 0.035 ± 0.027 *vs* 0.030 ± 0.024, p=0.016) after the consumption of the synbiotic compared to baseline values for *H. pylori*-positive subjects, while no changes were found in *H. pylori*-negative subjects (Figure 5). There were also some differences in changes of the ratio of oxidized to reduced glutathione during synbiotic and placebo treatment in *H. pylori*-positive subjects (mean –0.005 ± 0.011 *vs* –0.001 ± 0.011, p=0.099 (trend)). The decrease in the ratio was mainly due to the increase of GSH (972.1 *vs* 1018.1 μg/ml, p=0.063) in *H. pylori*-positive subjects.

In *H. pylori*-positive subjects the serum values of TAS were significantly lower compared to *H. pylori*-negative subjects (0.97 *vs* 1.05 mmol/l, p=0.008). After consumption of the synbiotic a beneficial influence was detected: TAS values increased in *H. pylori*-positive persons (0.97 *vs* 1.03 mmol/l, p=0.004). A similar increase in the values of TAS in the placebo period could have been due to the carryover effect on synbiotic consumption in the crossover study. No changes were found in the TAS values of *H. pylori*-negative persons.

Wnuk and co-workers have shown reduced antioxidative activity in the saliva of *H. pylori*-colonized persons (Wnuk *et al.*, 2010). Mashimo *et al*. (2006) have shown that elevated ROS of *H. pylori*-positive subjects in peripheral blood decreased after *H. pylori* eradication (Mashimo *et al.*, 2006). It

has been reported that after successful eradication of *H. pylori* oxidative stress in gastric mucosa was reduced (Pignatelli *et al.*, 2001; Katsurahara *et al.*, 2009).

Thus, consumption of the synbiotic containing the antioxidative probiotic *L. fermentum* ME-3 may improve reduced systemic antioxidative activity in *H. pylori*-colonized asymptomatic subjects. However, the results need to be confirmed in randomised larger studies of *H. pylori* colonized asymptomatic and diseased patients.

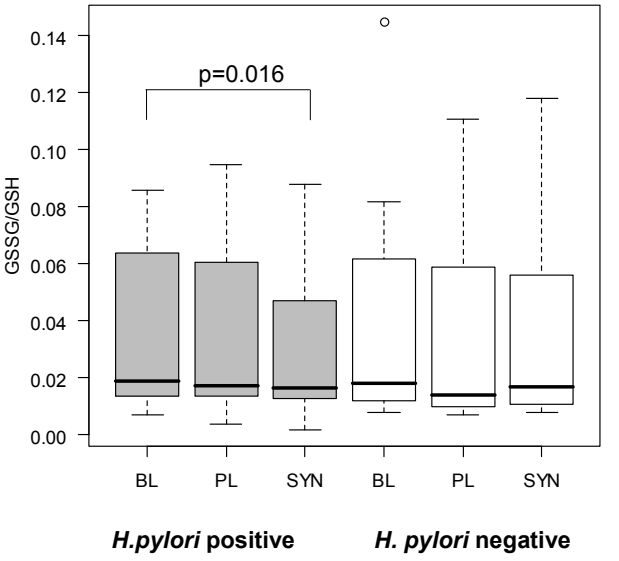


Figure 5. Changes in GSSG⁄GSH values during the trial in *H. pylori*-positive (n=28) and *H. pylori*-negative (n=25) individuals.

BL, baseline; Syn, end of synbiotic consumption; PL, end of placebo period.

5.4. Safety assessment

In our studies, according to the self-reported questionnaire, the study subjects tolerated the consumption of lactobacilli well although some individual differences were noted.

All haematological and functional indices of the liver and kidney remained in a normal range in all participants who completed the trial (Table 13).

There were no significant differences in BMI and biochemical indices (glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides) between the consumption of probiotic cheese and control cheese (Table 14).

The amount of cheese consumed during the study did not cause changes in the total cholesterol level of the participants, indicating the safety of the dosage of cheese (50 g daily). The dose of consumed cheese was even higher than that

used in a study of elderly persons without increased blood cholesterol after daily consumption of probiotic cheese (15 g, 10^9 CFU/day) for 4 weeks (Ibrahim *et al.*, 2010).

Thus, consumption of these *Lactobacillus* strains (Table 10) can be considered safe. This can be stated on the basis of the questionnaire results concerning abdominal symptoms without any serious adverse gastrointestinal complaints, as well as alterations in the reference ranges of the tested functional indices of the kidney and liver, and in inflammatory markers, and total blood cholesterol values.

Table 13. Blood inflammatory and functional indices of the kidney and liver assessed in human trials (expressed as mean ± SD).

Indices		**OLT**	**DBPCT TE1**	**DBPCT ELD**	**Reference values**
WBC × 10^9 L	B	6.0 ± 1.3	5.2 ± 0.8	4.9 ± 1.3	3.5–8.8 × 10^9/l
	A	6.1 ± 0.8	5.6 ± 1.3	4.8 ± 1.3	
hs-CRP (mg/L)	B	0.7 ± 0.4	1.1 ± 0.6	1.6 ± 1.9	< 5 mg/l
	A	0.8 ± 0.5	1.0 ± 0.3	1.8 ± 1.7	
ASAT (U/L)	B	24.9 ± 7.0	20.2 ± 5.4	21.3 ± 4.5	F < 32 U/l
	A	25.6 ± 6.4	18.3 ± 5.4	22.3 ± 4.6	M < 38 U/l
ALAT (U/L)	B	21.4 ± 7.5	17.7 ± 5.6	18.5 ± 6.1	F < 31 U/l
	A	25.4 ± 12.9	16.2 ± 4.3	21.5 ± 7.2	M < 41 U/l
Albumin (g/L)	B	43.1 ± 2.1	45.9 ± 2.6	41.8 ± 2.1	34–48 g/l
	A	44.3 ± 2.7	45.2 ± 1.8	41.8 ± 2.7	
Serum creatinine	B	76.7 ± 15.9	73.8 ± 12.0	64.5 ± 8.9	F < 81 μmol/l
(μmol/L)	A	77.6 ± 10.7	70.9 ± 10.3	64.2 ± 10.9	M < 107 μmol/l

B, before consumption; A, after consumption
hs-CRP, high sensitive C-reactive protein; ASAT, alanine aminotransaminase; ALAT, aspartate aminotransaminase, F, female, M, male
DBPCT, double-blind, placebo-controlled trial; placebo-controlled trial; OLT, open-label trial

Table 14. Anthropometric, clinical and biochemical parameters of the volunteers participating in the probiotic cheese trials (DBPCT) (expressed as mean ± SD).

Indices	Study	The probiotic cheese consumption period		The placebo cheese consumption period		p values (PRO *vs* PL)	Reference intervals for both genders by NORIP #
		At beginning	At end	At beginning	At end		
BMI (kg/m^2)	TE1	24.1 ± 3.6	24.2 ± 3.6	23.8 ± 3.5	23.9 ± 3.6	0.260	normal range 18.5–24.9, overweight 25–29.9, obese 30.0
	ELD	27.3 ± 4.2	27.3 ± 4.2	27.3 ± 4.1	27.4 ± 4.2	0.378	
Glu, mmol/l	TE1	4.5 ± 0.7	4.6 ± 0.5	4.6 ± 0.6	4.7 ± 0.5	0.486	≥ 18 yrs 3.3–5.5 mmol/l
	ELD	5.1 ± 0.5	5.4 ± 0.5	5.3 ± 0.5	5.4 ± 0.4	0.757	
Chol, mmol/l	TE1	4.6 ± 0.9	4.6 ± 1.1	4.2 ± 0.6	4.5 ± 0.9	0.671	30 – ≤ 50 yrs 3.3–6.9 mmol/l
	ELD	5.7 ± 0.8	5.6 ± 0.8	5.9 ± 0.9	5.7 ± 0.8	0.087	> 50 yrs 3.9–7.8 mmol/l
LDL, mmol/l	TE1	2.7 ± 0.8	2.8 ± 1.1	2.6 ± 0.7	2.6 ± 0.7	0.271	30 – ≤ 50 yrs 1.4–4.7 mmol/l
	ELD	3.9 ± 0.8	3.8 ± 0.7	4.1 ± 0.9	3.8 ± 0.7	0.919	≥ 50 yrs 2.0–5.3 mmol/l
HDL, mmol/l	TE1	1.7 ± 0.5	1.7 ± 0.3	1.6 ± 0.4	1.7 ± 0.4	0.623	≥ 18 yrs: 1.2 mmol/l
	ELD	1.7 ± 0.4	1.6 ± 0.4	1.7 ± 0.4	1.7 ± 0.5	0.118	
TG, mmol/l	TE1	1.0 ± 0.6	1.0 ± 0.5	0.9 ± 0.4	1.2 ± 0.7	0.428	≥ 18 yrs 0.45–2.6 mmol/l
	ELD	1.1 ± 0.6	1.1 ± 0.6	1.2 ± 0.5	1.1 ± 0.5	0.097	

BMI, body mass index; Glu, glucose, Chol, total cholesterol, LDL, LDL-cholesterol; HDL, HDL-cholesterol; TG, triglycerides # Intervals for routine laboratory tests proposed by Nordic Reference Interval Project (NORIP, http://www.furst.no/norip/) were used as the reference.

5.5. Survival and persistence of consumed probiotics

Main preconditions for probiotic impact are the ability to survive and persist in the GI environment and to reach high numbers of viable counts in the targeted part of the gut after consumption of a probiotic product. Faecal recovery of orally administered probiotic strains is a standard method to find out survival and persistence in the gastrointestinal tract. It has not been shown earlier how the results obtained by *in vitro* tests of tolerance of lactobacilli could predict the behaviour of these particular strains *in vivo*.

Manifestations of the colonizing properties of putative probiotics in human organism

We used cultural and molecular methods to determine the survival and persistence in the GI tract after consumption of the five putative probiotic strains. *L. acidophilus* 821-3 showed the highest score of colonizing potential (auto-aggregation ability, acid, bile and pancreatin tolerance) *in vitro* (Table 5). It survived and persisted well in the human gut after consumption of mixture of five different putative probiotics in capsule form (Table 15).

Although *L. gasseri* E16B7 showed good viability in simulated GI tract conditions, this strain survived but did not persist in the GI tract of healthy volunteers (paper IV). The finding indicates that persistence of lactobacilli in the GI tract may be influenced additionally by several other factors such as peristalsis, diet, and interactions with the indigenous intestinal microbiota.

Despite the lowest *in vitro* data (no auto-aggregation ability) of the colonizing potential of the probiotic *L. plantarum* TENSIA (Table 5) the strain survived well in cheese and therefore was chosen for human studies. In clinical studies, *L. plantarum* TENSIA was detected in faecal samples of five out of altogether 30 adults and healthy adults aged over 60 years even after 5 weeks of cessation of cheese consumption (Table 16).

Several earlier studies have demonstrated that dairy products used as vehicles for probiotics enhance the tolerance for GI transit (Stanton *et al.*, 2005). An example is cheese, where the anaerobic environment, high fat content and buffering capacity of the matrix helps to protect the probiotic cells both in the product and in the intestinal environment.

The results of our study suggested that the colonizing potential of putative probiotics were strain-specific, which is in agreement with some other studies (Mishra and Prasad, 2005). The favourable colonizing properties of lactobacilli detected in *vitro* experiments should still be confirmed in human studies with and without carrier vechicles.

Comparison of different methods for detection of putative probiotics

In our study we used a conventional plating method with typing AP-PCR and/or real-time PCR for detection of consumed *Lactobacillus* strains in faeces.

We compared the prevalence and counts of the strain *L. acidophilus* 821-3 obtained by real-time PCR assay and by conventional cultivation followed with AP-PCR (Table 15).

There was no difference in the prevalence of *L. acidophilus* 821-3 using both methods but the counts of *L. acidophilus* 821-3 determined by real-time PCR were significantly higher (up to 2 $\log_{10}$ CFU higher) (Table 15).

These data are in good accordance with previous studies on the topic (Ahlroos and Tynkkynen, 2009; Dommels *et al.*, 2009). Real-time PCR methods quantify bacterial loads inferred from the number of copies of a particular DNA target sequence. Thus, this method does not differentiate between dead and live bacteria, which lead to an overestimation of the number of viable cells. Furthermore, in relation to conventional cultivation methods, not all colonies may originate from a single viable cell, which leads to an underestimation of the number of viable cells. In order to obtain a more accurate measurement of viable cells using quantitative PCR, Cenciarini-Borde *et al.* (2009) have suggested treating of bacteria with propidinium or ethidium monoazide prior to the DNA isolation for selective suppression of amplification of DNA released from dead cells (Cenciarini-Borde *et al.*, 2009).

There was a significant difference between the two methods for evaluation of the prevalence of *L. plantarum* TENSIA in over 60-year-old persons. Namely, *L. plantarum* TENSIA was more often detected in case of real-time PCR compared to cultivation with AP-PCR typing (presence/absence of strain – 0/18 *vs* 9/9, p=0.001) 2 weeks after cessation of consumption of probiotic cheese (Table 16).

Surprisingly, in case of *L. plantarum* TENSIA, the counts obtained by real-time PCR were significantly lower compared to counts obtained by cultivation with AP-PCR typing (median 4.2 cell/g *vs* 6.3 $\log_{10}$ CFU/g p=0.011) in 60-year-old persons at the end of the consumption of the probiotic (Table 16). This may be considered a technical error due to repeated melting of the faecal samples for different sets of experiments.

Table 15. Prevalence and counts of recovered *L. acidophilus* 821-3 (counts in $\log_{10}$ cfu/g or cell/g in range, median).

Study			Consumption of putative probiotics		Cessation of consumption of putative probiotics	
			At beginning	At end (Day 5)	Day 8	Day 20
Phase 1* n=9	A	Prevalence	0 / 9	6 / 3	4 / 5	0 / 9
	B		1 / 8	9 / 0	7 / 2	1 / 8
	A	Counts, $\log_{10}$ cfu/g	0	0–6.8 (5.8)	0–7.0 (0)	0
	B	cell/g	0–5.2 (0)	6.7–8.7 (8.0)	0–8.2 (5.4)	0 – 5.5 (0)
Phase 2 n=5	B	Prevalence	0 / 5	5 / 0	5 / 0	1 / 4
		cell/g	0	6.7–8.7 (5.0)	5.3–8.4 (6.0)	0–5.1 (0)

* Mixture of 5 strains: *L. acidophilus* 821-3, *L. paracasei* 317, *L. fermentum* 338-1-1, *L. gasseri* 177, *L. gasseri* E16B7

Prevalence, presence /absence; A, cultivation with typing AP-PCR; B, real time PCR

Table 16. Prevalence and counts of recovered *L. plantarum* TENSIA (counts in $\log_{10}$ cfu/g or cell/g in range, median).

Studies			Consumption of putative probiotics		Cessation of consumption of putative probiotics	
			At beginning	At end	At beginning	At end
TE1 n=12	A	Prevalence	0 / 12	10 / 2	2 / 10	1 / 11
		Counts	0	0–8.6 (5.9)	0–4.3 (0)	0–2.6 (0)
ELD n=18	A	Prevalence	0 / 18	15 / 3	0 / 18 #	0 / 18
	B		0 / 18	11 / 7	9 / 9 #	4 / 14
	A	Counts, $\log_{10}$ cfu/g	0	0–8.3 (6.3) *	0	0
	B	cell/g	0	0–7.6 (4.2) *	0–7.9 (4.2)	0–5.3 (0)

Prevalence, presence /absence; A, cultivation with typing AP-PCR; B, real time PCR
A significant difference in prevalence of TENSIA using two different methods (p=0.001).
* A significant difference in counts of TENSIA using two different methods (p=0.011).

Different administered doses of putative probiotic

The *L. acidophilus* 821-3 was administered in high doses (1×10^{10} CFU) and the strain was detected by molecular methods at the end (Day 5) in all persons who had consumed this strain (Table 15).

Saxelin and co-workers have showed that the effective dose for survival and persistence is higher than 10^9 CFU per day. They suggested that the effective

daily dose should be $10^{10–11}$ CFU (Saxelin *et al.*, 1991; Saxelin *et al.*, 1995). Thus, our data are in good concordance with Saxelin's findings.

In a previously conducted randomised, double-blind, placebo-controlled trial the consumption of capsules of *L. fermentum* ME-3 in daily dose 1.5×10^9 CFU affected the total count of lactobacilli and the population of *L. fermentum*. However, *L. fermentum* ME-3 was not detectable at the end of consumption in faecal samples using classical cultivation methods (Songisepp *et al.*, 2005).

Multi-strain or species combination-based probiotics

Timmermann *et al* (2004) has suggested that multispecies probiotics may express improved functionality as compared to single strain probiotics (Timmerman *et al.*, 2004).

Nevertheless, we found that *L. acidophilus* 821-3 survived and persisted in the GI tract in both cases when administered as a single strain or in a mixture.

5.5.1. Changes in counts of faecal lactobacilli

Our study also demonstrated that administration of *Lactobacillus* multispecies strains changed the counts of faecal lactobacilli (Table 17). Consumption of capsules containing a mixture of five putative probiotic strains (*L. acidophilus* 821-3, *L. paracasei* 317, *L. fermentum* 338-1-1, *L. gasseri* 177 and E16B7) in high doses (5×10^{10} CFU/daily) increased significantly the total intestinal lactobacilli count obtained by cultivation (median lactobacilli counts from 5.0 to 6.6 $\log_{10}$ CFU/g; p=0.014) and by real-time PCR (from 7.2 to 8.2 cell/g; p=0.008).

At the same time, *L. acidophilus* 821-3 ingested as a monostrain (1×10^{10} CFU/daily) did not have a similar effect (median lactobacilli count from 7.7 to 7.6 cell/g, p=0.625) analysed by real-time PCR (Table 17).

The consumption of probiotic cheese comprising *L. plantarum* TENSIA for 3 weeks increased total faecal lactobacilli counts in both study groups (median lactobacilli count from 5.9 to 6.7 $\log_{10}$ CFU/g, p=0.047 and from 5.7 to 6.3 CFU/g, p=0.009, respectively).

No increase in total lactobacilli counts was seen in the placebo period (Table 17).

Mutual interactions take place between a probiotic strain and the host's indigenous microbiota in the small intestine. Some studies showed that ingestion of a certain probiotic may increase the total number of indigenous lactobacilli (Sepp *et al.*, 1993; Goossens *et al.*, 2003). In a previous trial, the consumption of a lower dose (1.5×10^9 CFU/daily) of *L. fermentum* ME-3 in capsules increased significantly the counts of total faecal lactobacilli (by 1.3 $\log_{10}$ CFU/g, p=0.023) (Songisepp *et al.*, 2005).

Table 17. Changes in the counts of total lactobacilli (log_{10} CFU/g) during the trials (range, median).

Consumption of putative probiotic strains			**Consumption of putative probiotics**		**p values**	**Cessation of consumption of putative probiotics**		**p values**
Mixture of cultures (5 strains)*			**At beginning**	**At end (Day 5)**		**Day 8**	**Day 20**	
n=14	A	Counts, log_{10} CFU/g	0–6.3 (5.0)	6.0–8.3 (6.6)	0.014	0–7.3 (5.3)	0–7.3 (4.6)	0.888
	B	cell/g	6.6–8.1 (7.2)	6.9–9.0 (8.2)	0.008	0–8.7 (7.0)	6.3–8.0 (7.2)	0.910
L. acidophilus 821-3 n=5	B	cell/g	7.4–8.6 (7.7)	6.8–9.3 (7.6)	0.625	6.7–9.3 (7.2)	0–8.9 (8.2)	0.813
***L. plantarum* TENSIA**			**Consumption of probiotic cheese**			**Consumption of placebo cheese**		
			At beginning	**At end**		**At beginning**	**At end**	
TE1 n=12	A	Counts, log_{10} CFU/g	2.8–6.3 (5.9)	4.9–8.6 (6.7)	0.047	4.0–8.6 (5.3)	4.3–7.8 (6.2)	0.213
ELD n=18	A	Counts, log_{10} CFU/g	0–8.3 (5.7)	4.3–10.0 (6.3)	0.009	0–9.7 (6.4)	0–9.1 (6.3)	0.583

*mixture of 5 strains: *L. acidophilus* 821-3, *L. paracasei* 317, *L. fermentum* 338-1-1, *L. gasseri* 177, *L. gasseri* E16B7
Counts of recovered putative probiotic strains using a conventional plating method with typing AP-PCR (A) and using real-time PCR (B).

6. GENERAL DISCUSSION

For elaboration of a particular probiotic the FAO guidelines are recommended. However, up to now it has not been assessed if the *in vitro* detected functional properties and markers of the colonizing potential of particular microbial strains are expressed in clinical trials with healthy volunteers. The present thesis was aimed to compare the required properties of the probiotic strains obtained by *in vitro* and animal experiments, and their impact on health indices of the human organism after consumption.

First, we aimed to assess the relationship between particular functional properties of 7 commercial probiotics and their manifestation in an efficacy study using a novel synbiotic, including the selected strains and a prebiotic.

Second, we aimed to screen the six putative *Lactobacillus* sp probiotics from the Human Microbiota Biobank (Acronym: HUMB registration number: 977) of the Department of Microbiology of the University of Tartu. The colonizing potential of *Lactobacillus* spp. and safety *in vivo* allowed to evaluate the properties required for the development of a probiotic. Next, the survival, persistence and safety of consumption of these putative probiotic strains during healthy volunteer trials were assessed.

The functional properties (*e.g.* antagonistic and antioxidative activity) of commercial probiotics were measured using *in vitro* tests. We found that the probiotic strain *L. fermentum* ME-3 expressed high antioxidative activity. *B. longum* 46 and *L. paracasei* 8700:2 showed good antimicrobial activity but differences in the suppression of GI pathogens were evident. Three strains (*B. longum* 46, *L. paracasei* 8700:2 *and L. fermentum* ME-3) were selected for the further synbiotic trial for evaluation of the expression of the functional properties in the human organism. In present dissertation we report the efficacy of synbiotic consumption reducing the oxidative stress related marker BDC-LDL of blood sera.

In our study six putative probiotic lactobacilli strains (*L. gasseri* 177, *L. gasseri* E16B7*, L. acidophilus* 821-3, *L. paracasei* 317, *L. fermentum* 338-1-1 and *L. paracasei* 1-4-2A) originated from faecal samples of healthy Estonian and Swedish infants. The colonizing potential (auto-aggregation ability, acid, bile and pancreatin tolerance) of the putative probiotics were evaluated in *in vitro* tests. All six strains were considered safe according to the results of *in vitro* tests (no haemolytic activity, no transferable antibiotic resistance). For evaluation of the translocation ability of the putative probiotis an animal model was used. The translocated strain *L. paracasei* 1-4-2A was excluded from further human trial as a non-safe strain for human consumption. The conducted human safety trials showed that consumption of five putative probiotic strains and of the probiotic *L. plantarum* TENSIA in cheese was safe and well tolerated according to to the self-reported questionnaire and normal blood indices. All five putative probiotics in capsules and *L. plantarum* in cheese maintained their viability and occured in sufficient numbers of live bacteria.

In summary, evaluation of probiotics has been based on general and functional aspects (Sanders and Huis in't Veld, 1999) (Figure 6). We have modified this scheme according to the results of the present PhD dissertation. We differentiated the functional properties for a particular strain and the colonizing potential, which are necessary for every probiotic strain. Basing on different studies, it was possible to reveal the association between the particular *in vitro* and animal experiments and the clinical results in humans.

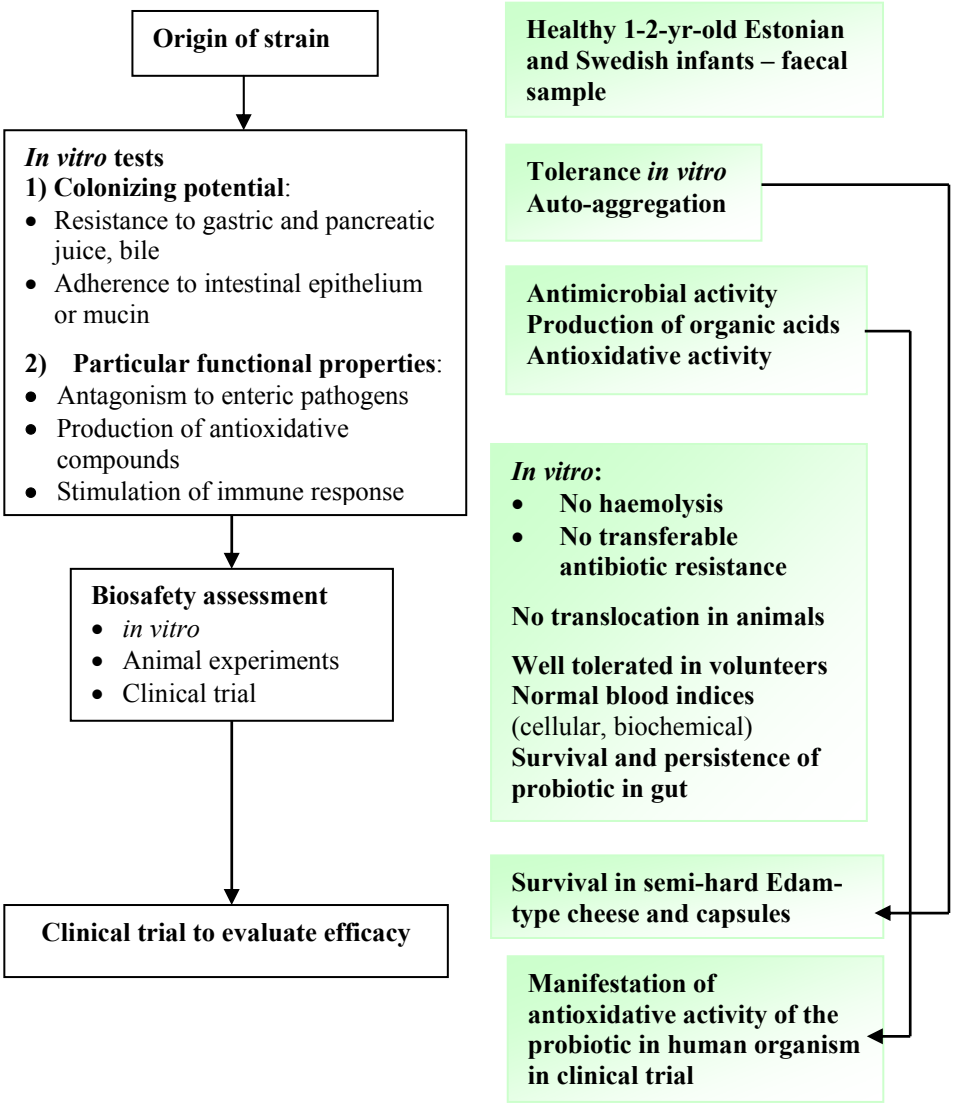


Figure 6. Summary of the evaluation of putative and commercial probiotic strains in the present dissertation. Green boxes indicate assessed properties of probiotic lactobacilli and bifidobacteria *in vitro* and *in vivo*. Arrow lines show the detected associations between *in vitro* and human studies.

6.1. Functional properties of probiotics

Evaluation of the antioxidative and antagonistic activity of already commercially available probiotics (five probiotic lactobacilli: *L. rhamnosus* GG, *L. fermentum* ME-3, *L. acidophilus* La5, *L. plantarum* 299v, *L. paracasei* 8700:2 and of the two probiotic bifidobacteria: *B. lactis* Bb12, *B. longum* 46) against particular enteric and urinary pathogens (*Salmonella enterica* ssp. *enterica*, *Shigella sonnei*, *H. pylori*, *C. difficile* and uropathogenic *E. coli*) in different experimental conditions was performed according to the FAO regulations.

We evaluated the inhibition of pathogens by probiotics in conditions resembling the GI niche and found that the uropathogenic *E. coli* was highly suppressed by *L. rhamnosus* GG and both bifidobacteria strains. *Lactobacillus* strains *L. paracasei* 8700:2, *L. plantarum* 299v and *L. fermentum* ME-3 were the most effective against *Salmonella enterica* ssp. *enterica* in a microaerobic milieu while *L. fermentum* ME-3 and both bifidobacteria expressed high activity against *Shigella sonnei* in an anaerobic milieu. Inhibitory activity of all tested probiotics against *C. difficile* was low. *B. longum* 46, *L. paracasei* 8700:2, *L. rhamnosus* GG and *L. plantarum* 299v strains showed moderate antagonistic activity against *H. pylori* under microaerobic conditions on solid media (Figure 7).

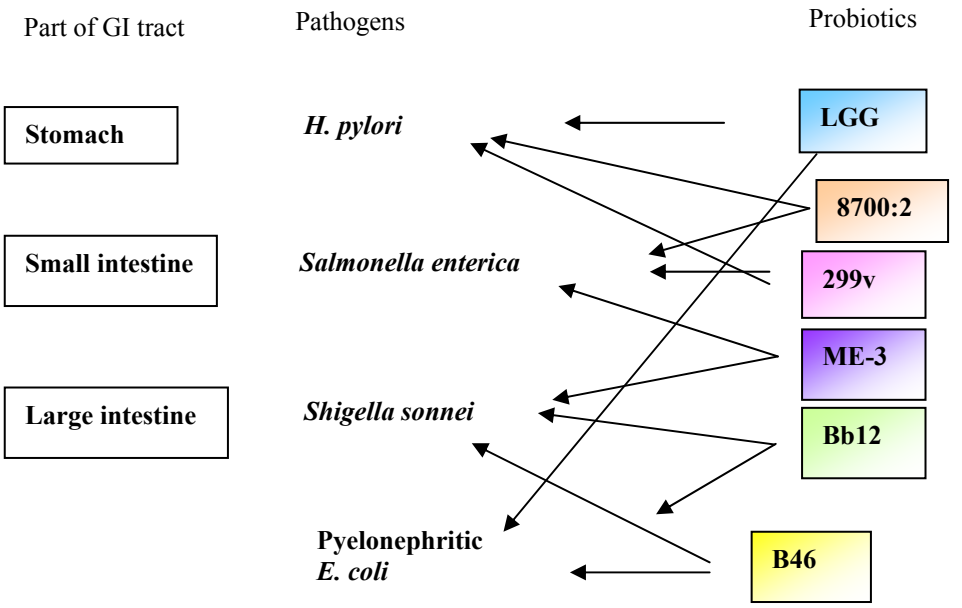


Figure 7. High suppression of different pathogens by 6 commercial probiotics. ME-3, *L. fermentum* ME-3; GG, *L. rhamnosus* GG; 299v, *L. plantarum* 299v; 8700:2, *L. paracasei* 8700:2; Bb12, *B. lactis* Bb12, B46, *B. longum* 46

The functional properties differ significantly among various *Lactobacillus* species and strains (Annuk *et al.*, 2003; Kõll *et al.*, 2008) Therefore, the functional characteristics of a *Lactobacillus* species or strain cannot be extrapolated to the other species or strains without evaluation.

An important functional characteristic of probiotic is antimicrobial activity which is based on production of different antimicrobial compounds. In general, the antimicrobial metabolites produced by probiotics can be divided into two groups: (i) low molecular mass compounds (bellow 1,000 Da) such as organic acids, which have a broad spectrum of action, and (ii) antimicrobial proteins, termed bacteriocins (>1.000 Da), which have a relatively narrow specificity of action against closely related organisms and other gram-positive bacteria (Ouwehand and Vesterlund, 2004).

Several different *in vitro* screening techniques (*e.g.* co-cultured in broth media, agar media using for spot-on-lawn assay, well diffusion assay and spot and streak line assay) for testing antimicrobial activity of probiotic candidates have been applied (Drago *et al.*, 1997; Jacobsen *et al.*, 1999; Annuk *et al.*, 2003; Moraes *et al.*, 2010).

It has been shown that the production of organic acids and antimicrobial activity of intestinal lactobacilli depend on their growth in different environments (Annuk *et al.*, 2003). Also biotope and fermentation type of lactobacilli may affect antagonistic activity towards pathogens in different environmental conditions (Annuk *et al.*, 2003). In addition, it is important to know the niche of a particular pathogen in the GI tract.

Our study showed that the antagonistic activity of a probiotic strain may vary in different growth media and in various environmental conditions. At the same time, it is important to know the niche of a particular pathogen in the GI tract to select probiotic strains with high antagonistic properties in similar environmental conditions. The antimicrobial activity of *L. fermentum* ME-3 has been confirmed in animal studies (Truusalu *et al.*, 2004; Truusalu *et al.*, 2008; Truusalu *et al.*, 2010). In healthy volunteers, *e.g.* the targeted group consuming functional food, it is complicated to test suppression of different pathogens. Thus, the evaluation of antimicrobial activity of probiotics against different pathogens in an appropriate *in vitro* environment should precede clinical efficacy studies.

There was some evidence that lactic acid bacteria (including lactobacilli and bifidobacteria) had an anti-oxidative potential (Kaizu *et al.*, 1993; Kullisaar *et al.*, 2002; Koller *et al.*, 2008; Kaushik *et al.*, 2009). This antioxidative property could favour lactobacilli to colonize the intestines, to protect the intestinal mucosa against excessive oxidative stress in the course of inflammation (Truusalu *et al.*, 2004; Saulnier *et al.*, 2011).

We found that the probiotic strain *L. fermentum* ME-3 expressed high antioxidative activity. *B. longum* 46 and *L. paracasei* 8700:2 showed good antimicrobial activity. Therefore, the three strains (*B. longum* 46, *L. paracasei* 8700:2 *and L. fermentum* ME-3) were selected for a further synbiotic trial.

6.2. Impact of functional properties of probiotics on human health

A number of probiotic species and strains used in clinical trials involving human participants, under a variety of conditions, have demonstrated efficacy. Recommendations for probiotic use in overtly healthy and diseased population according to clinical evidence have been proposed (Floch *et al.*, 2011).

To be effective, probiotic strains must retain the selected functional characteristic. Food carriers of probiotics may contribute to their survival in the GI tract. *Lactobacillus fermentum* ME-3 in spread cheese has prevented the oxidative spoilage of soft cheese products and may act as a natural antioxidant in soft cheese spreads with different fats (Jarvenpaa *et al.*, 2007).

Probiotics have also been used as adjunct to standard therapy for *H. pylori* eradication in clinical trials (Tursi *et al.*, 2004; Tong *et al.*, 2007). *H. pylori* infection causes both local and systemic oxidative stress in patients and, at the same time, *H. pylori* colonization affects negatively the antioxidative defence system (Jung *et al.*, 2001; Mashimo *et al.*, 2006). Previous reports indicate a decline in the levels of the endogenous cellular antioxidant GSH in gastric mucosa; also the decrease of the amount of ascorbic acid in serum and gastric juice in *H. pylori*-positive persons (Banerjee *et al.*, 1994; Verhulst *et al.*, 2000; Jung *et al.*, 2001; Shirin *et al.*, 2001). GSH is characterized as the cellular redox buffer acting as a scavenger of free radicals and toxic substances, and serving as a co-substrate for detoxification enzymes (Hansen *et al.*, 2009).

Reduction of oxidative stress by probiotics owing to their antioxidant properties has been demonstrated using animal models and human trials (Naruszewicz *et al.*, 2002; Kullisaar *et al.*, 2003; Truusalu *et al.*, 2004; Wang *et al.*, 2009; Truusalu *et al.*, 2010; Kullisaar *et al.*, 2011). Reduction of oxidative stress can be assessed by measuring the decrease of oxidative stress markers and/or increase of antioxidative defence markers.

In our previous study, antioxidative defence markers (TAA, TAS) of the blood of healthy volunteers increased significantly after 3-week consumption of *L. fermentum* ME-3 as a monostrain (Songisepp *et al.*, 2005).

Consumption of a dairy product comprising *L. fermentum* ME-3 has demonstrated reduction of oxidative stress in healthy volunteers. Namely, a 3-week consumption of goat milk fermented with ME-3 significantly improved the oxidative status (lowered BDC-LDL level in the plasma lipoprotein fraction, diminished level of oxLDL and suppressed production of 8-isoprostanes) and enhancement of TAS in the blood of humans (Kullisaar *et al.*, 2003). A very recent study showed that 2-week consumption *L. fermentum* ME-3 kefir reduced postprandial oxidative stress (decreased oxLDL, BDC-LDL and 8-isoprostanes) and decreased postprandial blood triglyceride level in clinically healthy subjects (Kullisaar *et al.*, 2011).

Some evidence suggests that treatment with *L. fermentum* ME-3 decreases oxidative stress in particular diseases *e.g.* atopic dermatitis and brain stroke. A

recent study showed that 3-month consumption of *L. fermentum* ME-3 fermented goat milk improved skin and blood antioxidative markers (GSSG/GSH ratio, total antioxidative capacity (TAC)), and decreased blood oxLDL levels in patients with atopic dermatitis (Kaur *et al.*, 2008). In addition, consumption of *L. fermentum* ME-3 capsules by brain stroke patients during rehabilitation improved their oxidative stress-related indices (GSSG/GSH, oxLDL) with significant positive correlation with clinical outcome according to Functional Independence Measure and Scandinavian Stroke Scale scores (Kullisaar *et al.*, 2008).

Thus, in our study of healthy persons the consumption of capsules containing a combination of different probiotic strains, including the antioxidative *L. fermentum* ME-3 strain with prebiotic raftilose P95, improved some general oxidative stress markers of blood. Evidently, the improved bioquality of LDL particles (BDC-LDL) and the decrease of the ratio of oxidized to reduced glutathione (GSSG/GSH) in *H. pylori*-positive subjects in the synbiotic trial were mainly due to the high antioxidative properties of *L. fermentum* ME-3.

6.3. Screening for the colonizing potential of putative probiotic strains in *in vitro* experiments

Functional requirements for probiotics should be established by using *in vitro* methods and the results of these studies should be reflected in controlled human studies. The colonizing potential of probiotics include tolerance to gastric acid, pancreatin and bile tolerance, and auto-aggregation, production of antimicrobial compounds to establish the niche in the biotope (Saarela *et al.*, 2000). *In vitro* tests examining maintenance of a strain's ability to tolerate acidic conditions in order to survive and grow in presence of bile and pancreatin can predict survival of the probiotic in the human organism (Dunne *et al.*, 2001).

In our study, all six tested putative probiotic strains and the probiotic *L. plantarum* TENSIA showed similar bile and pancreatin tolerance, while differences in acid tolerance and auto-aggregation were revealed. The results of our study suggest that the colonizing properties of *Lactobacillus* strains are strain-specific, which is in agreement with the findings of other studies (Mishra and Prasad, 2005; Delgado *et al.*, 2007). Similarly to our study, differences in the acid tolerance but also in the bile tolerance of intestinal *Lactobacillus* strains have been demonstrated (Delgado *et al.*, 2007).

The survival of probiotic strains with lower *in vitro* colonizing potential can be increased with suitable food carriers. Semi-hard cheese of Edam type proved to be an excellent carrier for *L. plantarum* TENSIA.

6.4. Safety assessment of putative probiotic strains *in vitro* experiments and in animal models

Despite many successful clinical studies and the long history of the use of lactobacilli, basic research for safety evaluation is important to ensure that probiotic strains have no potential risk. The FAO/WHO joint guideline has suggested several *in vitro* tests including correct taxonomic identification of probiotic strains, detection of the antibiotic resistance pattern and presence of antibiotic resistance genes, assessment of certain metabolic activities (*e.g.* d-lactate production) and testing for haemolytic activity for evaluation of the safety of probiotic microbes (FAO/WHO, 2002; Sanders *et al.*, 2010).

In our study the phenotypic identification of putative probiotic strains has been confirmed using molecular methods (arbitrarily primed PCR (AP-PCR) and by sequencing the 16S RNA gene.

Correct identification of probiotic strains to the species and strain levels are needed for strain selection and characterization, assessments of strain stability throughout the manufacturing process of the probiotic product, for proper description of the material used in human intervention studies, efficient tracking of the probiotic through the host, and for post-market surveillance including matching of strains isolated from any suspected infections (FAO/WHO, 2002; Sanders *et al.*, 2010).

Traditional methods (agar-plate cultivation, colony morphology, fermentation patterns) have been used for identification of probiotic strains. Unfortunately, the use of phenotypic tests is inadequate for species level resolution and the identification result should in any case be confirmed by molecular methods (Huys *et al.*, 2006; Vankerckhoven *et al.*, 2008).

All putative probiotic strains in our study were susceptible to ampicillin, gentamicin, erythromycin, tetracycline, chloramphenicol and rifampicin. The absence of phenotypic antimicrobial resistance of the tested *Lactobacillus* strains suggests the absence of transferable acquired resistance among these intestinal isolates.

Several studies provide the evidence of resistance to erythromycin and/or tetracycline among human faecal *Lactobacillus* isolates (Delgado *et al.*, 2007). In the last decade, there has arisen concern that the microorganisms used in food can be vehicles for transmission of antibiotic resistance genes (EFSA, 2008). Therefore, safety assessment of *Lactobacillus* strains should include detection of their antibiotic resistance profile (Bernardeau *et al.*, 2008).

Some *Lactobacillus* strains may show alpha haemolysis around colonies on blood agar plates (Olano *et al.*, 2001). Detection of the haemolytic potential of putative probiotics is required for safety reasons (FAO/WHO, 2002). According to our results, the selected putative probiotic strains did not have haemolytic capacities. This finding is accordance with a previous study showing non-haemolytic activity among lactobacilli of dairy and human origin (Maragkoudakis *et al.*, 2009).

Bacterial translocation is one of the most serious issues of probiotic safety. It is important to evaluate the translocation ability of putative probiotics using an animal model. It is well known that translocation of administered probiotics or commensal *Lactobacillus* strains may induce infections including bacteremia, sepsis, or endocarditis (Liong, 2008). It is important to evaluate the translocation ability of putative probiotics using a suitable animal model.

In our study we used two different lines of experimental mice with similar results. Five out of the 6 tested putative probiotic strains did not cause bacterial translocation. Only, *L. paracasei* strain 1-4-2A caused bacterial translocation to the spleen. The translocated strain *L. paracasei* 1-4-2A was excluded from further human trial as a non-safe strain for human consumption.

The probiotic *L. plantarum* TENSIA did not cause bacterial translocation in the animal model. According to safety testing *in vitro* and in animal models, five putative probiotics of *Lactobacillus* strains and the probiotic *L. plantarum* TENSIA were suitable for further human trials.

6.5. Safety evaluation in human trial

The safety of probiotics should be confirmed in healthy volunteers in controlled clinical trials. Although many research tools based on animal models or *in vitro* techniques are available, data from studies of humans are preferred whenever possible.

In our studies, according to the self-reported questionnaire, the study subjects tolerated the consumption of capsulated putative probiotic lactobacilli and probiotic cheese comprising *L. plantarum* TENSIA well although some individual differences were noted. All haematological and functional indices of the liver and kidney, and inflammatory markers remained in a normal range in all participants who completed the trial. Thus, the consumption of all these putative probiotic strains can be considered safe, which corresponds to suggestions for their evaluation.

There is evidence showing that lactobacilli in infections have been isolated mainly from individuals with underlying health deficiencies (*e.g.* predisposing heart valve defects, cancer, associated infectious diseases, chronic inflammatory diseases, *etc.*) (Cannon *et al.*, 2005; Robin *et al.*, 2010; Sanders *et al.*, 2010; Kochan *et al.*, 2011). Moreover, a recent study in patients with severe acute pancreatitis, who received a multispecies probiotic preparation, had higher mortality compared to the placebo group (Besselink *et al.*, 2009). In most cases intestinal commensals were translocated.

When a probiotic is planned to be investigated for the first time in a specific patient group, it is recommended that preliminary pilot safety trials be undertaken, which include routine monitoring for adverse events (Whelan and Myers, 2010).

6.6. Evaluation of survival and persistence of putative probiotic strains in human organism

To be effective, probiotic strains must retain the functional characteristics and the colonizing potential for which they were originally selected. Such characteristics include the ability to survive transit through the stomach and small intestine and to colonize the human GI tract (Tuomola *et al.*, 2001).

Faecal recovery of orally administered probiotic strains is a standard method for survival and persistence in the GI tract. In general, a probiotic strain disappears from the GI tract 3 up to 14 days after cessation of probiotic consumption (Saxelin *et al.*, 1995; Alander *et al.*, 1999; Jacobsen *et al.*, 1999; Morelli *et al.*, 2003).

Survival and persistence may be influenced by the origin of lactobacilli and particular properties of the strain, as well as by parameters of the probiotic product (including food matrix) and features of the host, and by the methods used for enumeration (Walter, 2008; Sanders and Marco, 2010).

For determination of survival and persistence of probiotics in the GI tract cultural and molecular methods (*e.g.* real time PCR) have been applied (Songisepp *et al.*, 2005; Ahlroos and Tynkkynen, 2009; Dommels *et al.*, 2009; Saxelin *et al.*, 2010).

In our study we used both methods for determination of the survival and persistence of lactobacilli in the GI tract after consumption of *Lactobacillus* strains.

All five capsulated putative probiotic strains (*L. gasseri* 177 and E16B7, *L. paracasei* 317, *L. fermentum* 338-1-1, *L. acidophilus* 821-3) survived GI passage in humans and one of them, namely *L. acidophilus* 821-3, persisted even up to 10 days after cessation of consumption. According to *in vitro* tests, all tested putative probiotic strains showed similar bile and pancreatin tolerance, while differences in acid tolerance and auto-aggregation were detected. This indicates that survival and persistence is mostly influenced by acid tolerance and auto-aggregation.

The probiotic *L. plantarum* TENSIA survived well during GI transit and persisted even 5 weeks after cessation of the consumption of probiotic cheese. This confirmed good acid and bile tolerance obtained *in vitro* tests. Regardless of the absence auto-aggregation *in vitro*, *L. plantarum* TENSIA was able to persist for over two weeks.

6.7. Impact of consumption of putative probiotics on indigenous lactobacilli

Consumption of probiotics may influence the metabolism and population of the indigenous microbiota. Mutual interactions take place between a probiotic strain and the host's indigenous microbiota in the small intestine. Probiotics may

cause changes in the amount and composition of the indigenous microbiota (Songisepp *et al.*, 2005).

Very recently McNulty and co-workers have suggested that probiotic organisms are capable of altering the metabolic properties of a human microbial community. This was the result of altered gene expression in the microbiota induced by probiotics (McNulty *et al.*, 2011).

Some studies showed that ingestion of a certain probiotic may increase the total number of indigenous lactobacilli (Sepp *et al.*, 1993; Goossens *et al.*, 2003; Wind *et al.*, 2010). The increase of *Bifidobacterium* and *Lactobacillus* sp levels in the gut are correlated with numerous health endpoints. Microbiota changes due to probiotic intake include increased numbers of related phylotypes, decreasing pathogens and their toxins, stabilizing bacterial communities when perturbed (*e.g.* with antibiotics), or promoting a more rapid recovery from a perturbation (Sanders, 2011).

In our study the number of indigenous lactobacilli in faeces increased during administration of a mixture of putative probiotic strains or *L. plantarum* TENSIA but decreased again when administration stopped. When a particular *L. acidophilus* strain was administered alone the increase of indigenous lactobacilli was not seen. This indicates that the effect to increase counts of indigenous lactobacilli may be strain specific.

Thus, the *L. acidophilus* strain 821-3 with good colonizing potential serves as a promising candidate for biotechnological application. The safety and persistence of the probiotic strain *L. plantarum* TENSIA (EE patent 05340) has been approved in clinical trials in healthy adults and in over 60-year-old volunteers.

7. LIMITATIONS OF THE STUDY

This thesis has some limitations.

First, in our synbiotic trial the 53% prevalence of *H. pylori* colonized persons was an unexpected finding offering the possibility to test the antimicrobial activity of the synbiotic components against the pathogen. Unfortunately, the design of the trial with application of enterocoated capsules did not allow testing of antimicrobial activity against *H. pylori* in the gastric cavity. The remarkable finding was that the systemic antioxidative defence (TAS) values of blood in *H. pylori* colonized persons were lower than in *H. pylori*-negative subjects. Further, a trend for a positive reduction of GSSG/GSH values by *L. fermentum* ME-3 in *H. pylori* colonized persons was found, offering a possibility for its further testing in *H. pylori* patients with different eradication regimens supplemented with the antioxidative probiotic.

Second, in the case of detection of *L. plantarum* TENSIA in faecal samples, the counts obtained by real-time PCR were significantly lower at the end of the consumption of probiotic cheese compared to the conventional plating method with AP-PCR typing performed earlier. Probably, the quality of DNA changed due to multiple handling in different laboratories. This finding indicates the need for simultaneous use of different detection methods for same set of experiments.

Third, the sample size of volunteers for the safety trial was really small but pilot studies with strains still under investigation have always been suggested with a limited number of participants.

8. PRACTICAL APPLICATIONS

In summary, the thesis established several necessary steps for development of probiotics.

We showed that the antimicrobial activity of probiotics against gastrointestinal pathogens should be tested in different environments and conditions mimicking the distinct GI niches before application in humans.

The other functional properties, including antioxidative activity, should also be evaluated offering possibilities for influencing the oxidative stress related markers as novel approaches. Oxidative stress has been demonstrated as an important factor in the pathogenesis of several diseases.

Screening for putative probiotics *in vitro* and animal models is necessary to find out new putative probiotic strains. Safety assessment of a putative probiotic in a pilot study should precede randomised double-blind placebo controlled efficacy studies.

9. CONCLUSIONS

1) The 7 the commercial probiotic strains expressed *in vitro* variable antimicrobial activity against different pathogens. Namely, *L. paracasei* 8700:2, *L. plantarum* 299v and *L. fermentum* ME-3 suppressed *Salmonella enterica*, while *L. rhamnosus* GG, *L. plantarum* 299v and *L. paracasei* 8700:2 inhibited *Helicobacter pylori* – the pathogens residing in the small intestine and in the stomach, respectively. In the anaerobic environment of the lower GI tract *B. longum* 46, *B. lactis* Bb12 and *L. fermentum* ME-3 inhibited *Shigella sonnei*, while both bifidobacteria strains and *L. rhamnosus* GG expressed high activity against the pyelonephritic strain of *E. coli*, respectively. Evaluation of the antimicrobial activity of probiotics against different pathogens in an appropriate environment mimicking GI niches should precede clinical efficacy studies.
2) *L. fermentum* ME-3 expressed the highest antioxidative activity among the tested 7 commercial strains in *in vitro* experiments. Consumption of the synbiotic containing three commercial strains (antimicrobial *L. paracasei* 8700:2 and *B. longum* 46 and antioxidative *L. fermentum* ME-3) with prebiotic raftilose P95 decreased systemic oxidative stress manifested in the reduction of the circulating BDC-LDL values of the blood of volunteers, proving the antioxidative efficacy of the synbiotic.
3) Screening for the colonizing potential of *Lactobacillus* sp. (auto-aggregation, acid and bile tolerance) by *in vitro* experiments allowed selecting the most promising candidates for clinical trials with putative probiotic strains.
4) *In vitro* testing of *L. plantarum* TENSIA and of the putative 6 probiotic strains for absence of haemolytic activity and transferable antibiotic resistance confirmed their safe potential for human application. Additional estimation of possible translocation to different organs in an animal model helped to exclude the non-safe *Lactobacillus* strain (*L. paracasei* 1-4-2A) from the human study.
5) The putative probiotic strains tolerated well the passage through the GI tract. The persistence of a particular strain varied from 3 days up to 5 weeks after cessation of the consumption *Lactobacillus* strains. We suggest that the survival of probiotic strains with lower *in vitro* colonizing potential can be increased with suitable food carriers. The semi-hard cheese of Edam type was an excellent carrier for *L. plantarum* TENSIA.
6) The administration of the probiotic cheese comprising *L. plantarum* TENSIA and high doses of the capsulated *Lactobacillus* multistrains did not result in any serious adverse gastrointestinal complaints and/or abnormal values of blood indices. Thus, the safety of the capsulated *Lactobacillus* strains and the patented *L. plantarum* TENSIA strain, elaborated in probiotics was approved in clinical trials with healthy persons, as a basis for its application as functional food.

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11. SUMMARY IN ESTONIAN

Potentsiaalsete probiootiliste laktobatsillide funktsionaalsed omadused, püsivus, ohutus ja tõhusus

Inimese seedetrakti mikrobioota mõjutab erinevaid bioloogilisi funktsioone. Mikrobioota soodustab seedimist, soole epiteelirakkude küpsemist ning mõjutab erinevaid füsioloogilisi näitajaid, sh vere lipiidide hulka, inhibeerib kahjulikke baktereid ja stimuleerib immuunsüsteemi.

Vähenenud sündimus ja pikem eluiga on suurendanud krooniliste haiguste (nt südame-veresoonkonna haigused, erinevad ainevahetushäired) levimust. Lisaks on jätkuvalt olulisel kohal erinevad infektsioonid: respiratoorsed ja uro-infektsioonid ning toidu teel levivad infektsioonid, samas ka *Helicobacter pylori* infektsioon. Seetõttu vajab elanikkond haiguste ennetamiseks erinevaid meetmeid, sealhulgas tervislikumat toitumist.

Eelpoolnimetatud krooniliste haiguste (sh *H. pylori* infektsiooni) patogeneesis on olulisel kohal oksüdatiivne stress. Mõned piimhappebakterid (sh laktobatsillid ja bifidobakterid) omavad antioksüdatiivset aktiivsust, nt suudavad vähendada reaktiivsete hapnikuühendite kuhjumist inimorganismis, produtseerivad Mn-superoksiidi dismutaasi, lõhustavad hüdroksüülradikaale ja on võimelised alandama oksüdeeritud ja redutseeritud glutatiooni suhet (GSSG/GSH). Senini pole selliste omadustega piimhappebaktereid kasutatud funktsionaalse toiduna, et vähendada oksüdatiivset stressi inimorganismis ja sellest tulenevalt mõjutada haiguste kulgu.

Probiootikum on inimeselt pärinev elus mittepatogeenne mikroob, mille manustamine on tervisele kasulik. Probiootikumid leevendavad laktoositalumatuse sümptomeid, ennetavad toiduallergiat ja parandavad immuunsüsteemi võimekust ning taastavad mikrobioota koostist seoses antibiootikumraviga. Kuigi probiootikume peetakse üldiselt tervisele ohututeks, on iga potentsiaalset probiootikumi vaja põhjalikult testida. Vajalik on tagada probiootikumi tarbimise ohutus, püsimine seedekulglas ning tervistavate omaduste avaldumine inimorganismis füsioloogiliste ja biokeemiliste näitajate kaudu.

2002. aastal soovitasid FAO ja WHO astmelist skeemi probiootikumide hindamiseks. See skeem hõlmab vastava mikroobi üldist iseloomustamist *in vitro*, funktsionaalsete omaduste väljaselgitamist ja ohutuse tuvastamist loommudelil. Sellele järgneb tüve ohutuse ja tervistava toime hindamine inimestel kliinilistes uuringutes. Samas pole teaduspõhiselt tõestatud probiootikumide funktsionaalsete omaduste avaldumist kliinilistes uuringutes tervetel inimestel.

Uurimistöö eesmärgid ja ülesanded

Uurimistöö peamiseks ülesandeks oli hinnata kommertsiaalsete probiootikumide funktsionaalseid omadusi ning potentsiaalsete probiootiliste laktobatsillide koloniseerimisvõimet *in vitro* katsetes. Samas hinnata probiootikumide elulemust ja püsivust seedetraktis; probiootikumide ohutust ning efektiivsust tervetel täiskasvanutel ja üle 60-aastastel inimestel.

Uurimistöös püstitati järgmised ülesanded:

1. Hinnata kommertsiaalsete probiootiliste laktobatsillide (*L. rhamnosus* GG, *L. fermentum* ME-3, *L. acidophilus* La5, *L. plantarum* 299v, *L. paracasei* 8700:2) ja bifidobakterite (*B. lactis* Bb12, *B. longum* 46) entero- ja uropatogeenide vastast antagonistlikku toimet mikroaeroobses või anaeroobses keskkonnas, mis sarnaneb vastavale sooletrakti niššile ning määrata nende probiootikumide antioksüdatiivset aktiivsust *in vitro* katsetes.
2. Hinnata sünbiootikumi, mis sisaldab antimikroobsete ja antioksüdantsete omadustega kolme tüve (*L. fermentum* ME-3, *L. paracasei* 8700:2 ja *B. longum* 46) ja prebiootikumi raftiloos P95, tarbimise mõju vere oksüdatiivse stressi näitajale (oxLDL, BDC-LDL) tervetel inimestel.
3. Hinnata TÜ mikrobioloogia instituudi Inimese Mikrobioota Biopanga (akronüüm: HUMB, Registreerimisnumber: 977) laktobatsillide kollektsiooni tüvede koloniseerimisomadusi *in vitro* eksperimentidega, mis matkivad sooles olevaid tingimusi.
4. Määrata *L. plantarum* TENSIA ja potentsiaalsete probiootiliste tüvede (*L. gasseri* 177, *L. gasseri* E16B7*, L. acidophilus* 821-3, *L. paracasei* 317, *L. fermentum* 338-1-1, *L. paracasei* 1-4-2A) ohutust *in vitro* ja loomkatsetes.
5. Määrata *L. plantarum* TENSIA ja potentsiaalsete probiootiliste tüvede elulemust ja püsivust seedetraktis tervetel täiskasvanutel ning üle 60-aastatel isikutel.
6. Hinnata *L. plantarum* TENSIA ja potentsiaalsete probiootiliste tüvede tarbimise ohutust tervetel täiskasvanutel ning üle 60-aastatel isikutel.

Uuritav materjal ja meetodid

Erinevate kommertsiaalsete probiootiliste tüvede antagonistlikke omadusi uuriti erinevates kasvukeskkondades (mikroaeroobne ja anaeroobne) ja kahe erineva meetodi (agaril ja puljongis) abil järgnevate mikroobide suhtes: uropatogeenne *E. coli, Salmonella enterica* ssp. *enterica*, *Shigella sonnei*, *H. pylori* ja *C. difficile*. Probiootikumide metaboliite (äädik-, piim- ja merivaikhappe ning etanooli) määramiseks vedelsöötmes kasutati gaaskromatograafilist meetodit. Lisaks uuriti kommertsiaalsete probiootiliste laktobatsillide ja bifidobakterite antioksüdantseid omadusi *in vitro* eksperimentidega.

L. plantarum TENSIA ja 6 potentsiaalset probiootilist laktobatsilli tüve pärinesid eesti ja rootsi laste roojast. Määrati nende tüvede kolonisatsiooni-

võimet (auto-agregatsiooni, happe- ja sapitaluvust) ning ohutust (hemolüütilise aktiivsuse ning ülekantava antibiootikumresistentsuse puudumist). *In vitro* eksperimentidele järgnes ohutuse selgitamine loomkatsetes. *L. plantarum* TENSIA ja 5 sobilike omadustega potentsiaalset probiootilist tüve valiti välja edasiseks inimuuringuks, kus määrati nende tüvede tarbimise ohutust ning tüvede elulemust ja püsivust inimorganismis.

Probiootikumide funktsionaalsete omaduste avaldumist inimorganismis hinnati ühes kliinilises uuringus. Nimelt randomiseeritud topeltpimedas ümberlülitusega uuringus hinnati vere oksüdatiivse stressi näitajate muutust isikutel, kes tarbisid sünbiootikumi (*L. paracasei* 8700:2, *B. longum* 46 ja *L. fermentum* ME-3 koos prebiootikumi raftiloos P95) 3 nädalat.

Uurimistöö kokkuvõte

Erinevate potentsiaalsete probiootiliste bakterite koloniseerimisvõime määramiseks kasutati bakterioloogilisi, biokeemilisi ja molekulaarseid meetodeid. Potentsiaalsete probiootikumide ohutuse määramiseks tervetel vabatahtlikel kasutati kliinilises praktikas kasutusel olevaid vereanalüüse, antropomeetrilisi mõõtmisi ja ankeeti. Randomiseeritud topeltpimedas ümberlülitusega sünbiootikumi uuringus määrati vere oksüdatiivse stressi seotud näitajaid erinevate biokeemiliste testidega.

1. Viie kommertsiaalse probiootilise laktobatsilli ja kahe probiootilise bifidobakteri antimikroobne aktiivsus oli erinevates keskkondades erinev ning sõltus konkreetsest probiootikumist ja patogeensest mikroobist. Nimelt *L. paracasei* 8700:2, *L. plantarum* 299v ja *L. fermentum* ME-3 inhibeerisid *S. enterica*'t, mis asustab peensoolt ning *L. rhamnosus* GG, *L. plantarum* 299v ja *L. paracasei* 8700:2 surusid alla *H. pylori*'t, mis aga asustab magu. Anaeroobses keskkonnas, mis on sarnane jämesoolele, inhibeerisid *B. longum* 46, *B. lactis* Bb12 ja *L. fermentum* ME-3 patogeen *Shigella sonnei*'d ning mõlemad bifidobakterid ja *L. rhamnosus* GG surusid alla püelonefriiti tekitavat *E. coli'*t. Et hinnata probiootikumide antimikroobset aktiivsust erinevate patogeenide suhtes, mis asustavad inimese erinevaid seedetrakti piirkondi, tuleb antimikroobset aktiivsust määrata sobilike keskkondade tingimustes ning see peaks eelnema kliinilise efektiivsuse uuringutele.
2. *L. fermentum* ME-3 omas kõrget antioksüdatiivset aktiivsust võrreldes teiste kommertsiaalsete probiootikumidega *in vitro* eksperimentides. Kolme kommertsiaalse probiootilise tüve funktsionaalsete omaduste avaldumist (antimikroobsete *L. paracasei* 8700:2 ja *B. longum* 46 ning antioksüdatiivse *L. fermentum* ME-3) koos prebiootikum raftiloos P95-ga hinnati tervete täiskasvanute kliinilises uuringus. Sünbiootikumi tarbimine langetas süsteemset oksüdatiivset stressi, mis väljendus veres madala tihedusega lipoproteiinide dieenkonjugaatide (BDC-LDL) sisalduse vähenemises.
3. *In vitro* eksperimentidega, kus hinnatakse laktobatsillide koloniseerimisvõimet kirjeldavaid omadusi (autoagregatsioon, happe- ja sapitaluvus), on

võimalik kindlaks määrata kõige sobivamaid potentsiaalseid probiootilisi laktobatsille inimuuringuteks.

4. Tuginedes *in vitro* ohutuse testidele (hemolüütilise aktiivsuse ja ülekantava antibiootikumresistentsuse puudumine) olid kõik potentsiaalsed probiootikumid ja *L. plantarum* TENSIA ohutud. Vaid üks tüvi (*L. paracasei* 1-4-2A), mis oli ohutu *in vitro* katsetes, translotseerus hiire põrna. Seda tüve kui mitteohutut, inimuuringus ei kasutatud. See leid näitas, et on oluline määrata lisaks *in vitro* testidele ka ohutus loomkatses, vältimaks süsteemseid infektsioone mitteohutute laktobatsillide kasutamisest inimestel.
5. Potentsiaalsed probiootikumid säilitasid eluvõime pärast sooletrakti läbimist, mis on kooskõlas *in vitro* testide leiuga. *Lactobacillus* spp. püsivus sooletraktis erines tüveti ning kestis kolmest päevast kuni 5 nädalani pärast tüvede tarbimise lõpetamist. Madala elulemusega probiootiliste tüvede koloniseerimspotentsiaali saab tõsta kui kasutatakse sobivat toidumaatriksit probiootikumi kandjana. Poolkõva Edami tüüpi juust oli *L. plantarum* TENSIA'le sobilik kandja.
6. Erinevate kapsuleeritud laktobatsillide tüvede tarbimine kõrgetes doosides ja probiootikumina patenteeritud *L. plantarum* TENSIA tarbimine ei põhjustanud raskeid seedetrakti vaevusi ega ka olulisi muutusi verenäitajates. Seega kliiniliste uuringutega tervetel inimestel kinnitus uuritud tüvede ohutus, mis on aluseks nende kasutamiseks funktsionaalse toiduna.

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I would like to encourage my niece Veronica to graduate her studies at the University and also encourage brother-in-law Kari to start his PhD studies.

PUBLICATIONS

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Special courses

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The main subject of the research deals investigation of functional properties of probiotics and their manifestation in clinical trials. 12 scientific publications and 24 conference presentations.

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2002 –	TÜ arstiteaduskonna, Mikrobioloogia Instituudi doktorant

Teenistuskäik

Alates 1.sept. 1997	TÜ Mikrobioloogia Instituudi vanemlaborant
1. nov. 1997 – juuni 1998	TÜ arstiteaduskond, Mikrobioloogia Instituut, erakorraline assistent
2004 – ...	TÜ arstiteaduskond, Mikrobioloogia Instituut, Meditsiinilise mikrobioloogia ja viroloogia õppetool; vanemlaborant

Erialane täiendus

2002 Läbitud 1-nädalane kursus "Funktsionaalne toit ja vanurid" Soomes, Turu Ülikoolis

2003 Doktoriõppe raames läbitud bioeetika kursus

2004 Tutvusin VTT mikroorganismide kollektsiooniga Helsingis, Soomes.

2005 Tutvusin DSMZ'i mikroorganismide kollektsiooniga Braunschweigis, Saksamaal.

2010 Osalesin rahvusvahelisel seminaril (*5th International Workshop Nutrition & Health Claims Europe: Designing Clinical Studies for Success*) Brüsselis, Belgias

2011 Osalesin rahvusvahelisel seminaril (*6th International Workshop Nutrition & Health Claims Europe: Crisis or Opportunities?*) Brüsselis, Belgias

2011 Läbitud kursus "Kliiniliste uuringute meetodid"

Kutseorganisatsioonid

Eesti Laborimeditsiini Ühingu (ELMÜ) liige

Teadustegevus

Uurimisvaldkonnad: probiootiliste bakterite funktsionaalsete omaduste määramine ja probiootikumide tervist parandava toime välja selgitamiseks kliinilistes uuringutes. Uurimistööde alusel on avaldatud 12 teadusartiklit ja 24 konverentsiteesi.

Olulisemad publikatsioonid

Hütt P, Stsepetova J, Alvarez B, Mändar R, Krogh-Andersen K, Marcotte H, Hammarström L, Mikelsaar M. Safety and persistence of orally administered human *Lactobacillus* sp. strains in healthy adults. *Beneficial Microbes* 2011; 2: 79–90.

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Mikelsaar M, **Hütt P**, Kullisaar T, Zilmer K, Zilmer M Double benefit claims for antimicrobial and antioxidative probiotic. *Microbial Ecology in Health and Disease* 2008; 20(4): 184–188.

Songisepp E, Kals J, Kullisaar T, Mändar R, **Hütt P**, Zilmer M, Mikelsaar M. Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers. *Nutrition Journal* 2005; 4 (22): 141–146.

Maaroos H-I, Andreson H, Lõivukene K, **Hütt P**, Kolk H, Kull I, Labotkin K, Mikelsaar M. The diagnostic value of endoscopy and *H. pylori* tests for peptic ulcer patients in late posttreatment setting. *BMC Gastroenterology* 2003; 4(27): 1–6.

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