DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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The characterisation of intestinal lactic acid bacteria using bacteriological, biochemical and molecular approaches

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III Mikelsaar M, Annuk H, **Shchepetova J,** Mändar R, Sepp E, Björksten B. Intestinal lactobacilli of Estonian and Swedish children. *Microbial Ecology in Health and Disease* 2002; 14: 75–80.

IV **Štšepetova** J, Sepp E, Kolk H, Lõivukene K, Songisepp E, Mikelsaar M. Diversity and metabolic impact of intestinal *Lactobacillus* spp. in healthy adults and elderly. *British Journal of Nutrition* 2011; 105: 1235–1244. DOI: 10.1017/S0007114510004770

V **Štšepetova** J, Sepp E, Julge K, Vaughan E, Mikelsaar M, de Vos WM. Molecularly assessed shifts of *Bifidobacterium* ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. FEMS *Immunology and Medical Microbiology* 2007; 51: 260–269.

VI Mikelsaar M; Songisepp E; Smidt I, **Štšepetova J**; Zilmer M, Hütt P, Truusalu K, Kilk K. Isolated microorganism strain *Lactobacillus plantarum* Inducia DSM 21379 as probiotic enhancing natural immunity of organism, food product and composition comprising said microorganism and use of said microorganism for production of medicine for enhancing of cellular immunity. (Priority number: P200800027). PCT/EE2009/000006, Estonian Patent EE05341 B1, 07.09.2010.

Jelena Štšepetova has contributed to the following original publications:

Papers I, III: molecular analysis of lactobacilli, detection of metabolites by gas chromatography, participation in data analysis and manuscript drafting.

Paper II: study design, study performance, molecular analysis, data analysis and manuscript drafting.

Paper IV: study design, study performance, molecular analysis, data analysis and manuscript drafting.

Paper V: study design, study performance, molecular analysis, data analysis and manuscript drafting.

Paper VI: characterisation of *Lactobacillus plantarum* Inducia DSM 21379 by molecular methods, detection of metabolites by gas chromatography, data analysis.

ABBREVIATIONS

API 50CHL	Analytical Profile Index according to fermentation pattern of 50 Carbohydrates by *Lactobacillus* (isolates)
ADP	Adenosine Diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BMI	Body mass index
CAN	Columbia Agar with Colistin and Nalidixic Acids
CoA	Coenzyme A
CFU	Colony Forming Units
DAPI	4'6-diamino-2-phenylindole dihydrochloride
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DSM	Deutsche Sammlung von Mikroorganismen
EMP	Embden-Meyerhof-Parnas Pathway
F6PPK	Fructose-6-Phosphate Phosphoketolase
FAO	Food and Agriculture Organization
FHEL	Facultatively Heterofermentative Lactobacilli
FISH	Fluorescent *In Situ* Hybridization
GALT	Gut-associated Lymphoid Tissue
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
GI	Gastrointestinal Tract
HPLC	High Pressure Liquid Chromatography
ITS	Internal-Transcribed Spacer
ITS-PCR	Internal-Transcribed Spacer Polymerase Chain Reaction
LDH	Lactate Dehydrogenase
LAB	Lactic Acid Bacteria
LB	*Lactobacillus* spp.
MRS	de Man-Rogosa-Sharpe
NADH	Reduced Nicotinamide-adeninenucleotide
NAD	Nicotinamide-adeninedinucleotide
NO	Nitric oxide
NOS-NO	Nitric oxide synthase-nitric oxide system
NSP	Non-starch polysaccharides
ODS	Ornithine decarboxylase
OHEL	Obligately Heterofermentative Lactobacilli

OHOL	Obligately Homofermentative Lactobacilli
PFGE	Pulse Field Gel Electrophoresis
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SAM	S-adenosylmethionine
SCFA	Short-Chain Fatty Acids
TGGE	Temperature Gradient Gel Electrophoresis
WHO	World Health Organization

1. GENERAL INTRODUCTION

Lactic acid bacteria (LAB), including *Lactobacillus* spp. and *Bifidobacterium* spp., are well known and safe components of the microbial ecosystem in the gastrointestinal tract. They colonise the digestive tract soon after birth and are present in high numbers in both infants and adults (Mikelsaar and Mändar, 1993; Guarner and Malagelada, 2003). The potential role bifidobacteria in infant digestion (Tissier, 1900; Moro, 1900a, 1900b) and the application of lactobacilli-fermented food against the effects of aging and atherosclerosis have attracted attention since early in the last century (Metchnikoff, 1908). In more recent times, several investigations have connected the loss of LAB in the host with the development of allergies (Björksten et al., 2001; Kalliomäki et al., 2001). In the last 15 years the level of knowledge regarding LAB in the human host and its impact on human health has been exapnded considerably at the Department of Microbiology of Tartu University (Mikelsaar, 1993; Mändar, 1996; Karki, 1996; Naaber, 1997; Sepp, 1998; Annuk, 2002; Songisepp, 2005). However, there remains a lack of data regarding the species composition of intestinal LAB and their secreted metabolites in healthy and allergic children, and also during the aging process.

Worldwide, there is a rising demand for functional food incorporating probiotic bacteria as live microorganisms which administered in adequate amounts provide a health benefit to the host (FAO-Food and Agriculture Organization and WHO-World Health Organization, 2002). However, the information about which species and strains of LAB could provide the best health effect under different environmental conditions and at different age scale are still scarce. Although, the necessity of fundamental molecular typing of LAB probiotic candidates has been underlined (FAO; Vanckerhofen et al., 2008), the assessment of their molecular and metabolite profiles, which grant particular functional properties on the strains regarding health promotion, is often insufficient (Douglas and Sanders, 2008). The understanding of the complex genetic and metabolic properties of the microbiota is of the utmost importance for understanding and identifying candidate strains for novel group-specific and individual healthy diets.

The present thesis integrates data that has been collected over several years using available molecular (PCR, ITS-PCR, FISH, DGGE, real-time PCR**,** 16S rRNA sequencing) and biochemical methods (enzymatic profile, gas-chromatography) for characterisation of intestinal lactic acid bacteria from people in different age groups including people with allergies. Consequently, a well-characterised pool of lactobacilli strains has been created in the Department of Microbiology at the University of Tartu. The studies on metabolism of carbohydrates and polyamines have helped to reveal some novel functional properties of these collected lactobacilli. The author of the current PhD thesis participated in the charactertisation of the probiotic strain *Lactobacillus plantarum* Inducia (DSM 21379) which has been patented in Estonia (EE05341 B1, 07.09.2010)

and has an international patent application (PCT/EE2009/000006) filed. This probiotic aims to enhance natural immunity and the author has performed the molecular identification of the strain and evaluated its metabolic functional properties.

2. LITERATURE REVIEW

2.1. Human intestinal microbiota

2.1.1. Composition of intestinal microbiota

The human digestive tract harbours a wealth of different microbial ecosystems that vary according to their location within the intestinal tract. Culture-based studies have shown that faecal bacteria comprise between 400–500 distinct species and each individual human has a unique flora dominated by 30–40 species (Kimura et al., 1997). The application of molecular techniques has indicated that 60–80% of the organisms of the human microbiota have not been cultivated so far (Laugendijk et al., 1995; Saue et al., 1999; Eckburg et al., 2005). Based on molecular methods, current estimates indicate that intestinal microbiota consists of at least 10^{14} microbes and is dominated by anaerobic bacteria, comprising over 1000 species, among which gram-positive bacteria predominate (Vaughan et al., 2000; Zoetendal et al., 2004; Eckburg et al., 2005; Bäckhed et al., 2004; Ley et al., 2006; Ventura et al., 2009). The microbial composition and their relative proportions vary, depending on the physiological conditions of the compartment (e.g. pH), with different parts of the gastrointestinal tract, becoming richer and more diverse, ranging from the relatively germ-free stomach and the upper small intestine to the colon (Figure 1) (Kleessen et al., 1997). It has been demonstrated previously that the majority of faecal bacteria predominantly belong to genera such as *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Fusobacterium*, *Bifidobacterium* and *Peptostreptococcus* (Moore et al., 1974; Simon et al., 1984). Recent molecular studies have revealed that the gut microbiota of adults is largely dominated by the members of only two bacterial phyla, *Bacteroidetes* and *Firmicutes*, and a single member of the archaea, *Methanobrevibacter smithii* (Huys et al., 2008). More specifically, three bacterial groups predominate within these phyla: the *Bacteroides-Prevotella* group, the *Clostridium coccoides* group, and the *Clostridium leptum* group (Eckburg et al., 2005).

Lactobacillus spp. and *Streptococcus* spp. prevail in the microflora of small intestine (Finegold et al., 1983; Franks et al., 1998; Marteau et al., 2001; Zoetendal et al., 2006) whit a 10-fold difference in the ratio of anaerobes/ aerobes compared to a 1000-fold difference in caecum (Klessen et al., 2000; Macfarlane et al., 2000). The bacteria of the small intestine have the strongest influence on the immune system since the gut-associated-lymphoid-tissue (GALT) is situated mainly in the small intestinal and caecal mucosa (Schroff et al., 1995). Therefore, the composition and activity of the intestinal flora can have a profound influence on health and disease.

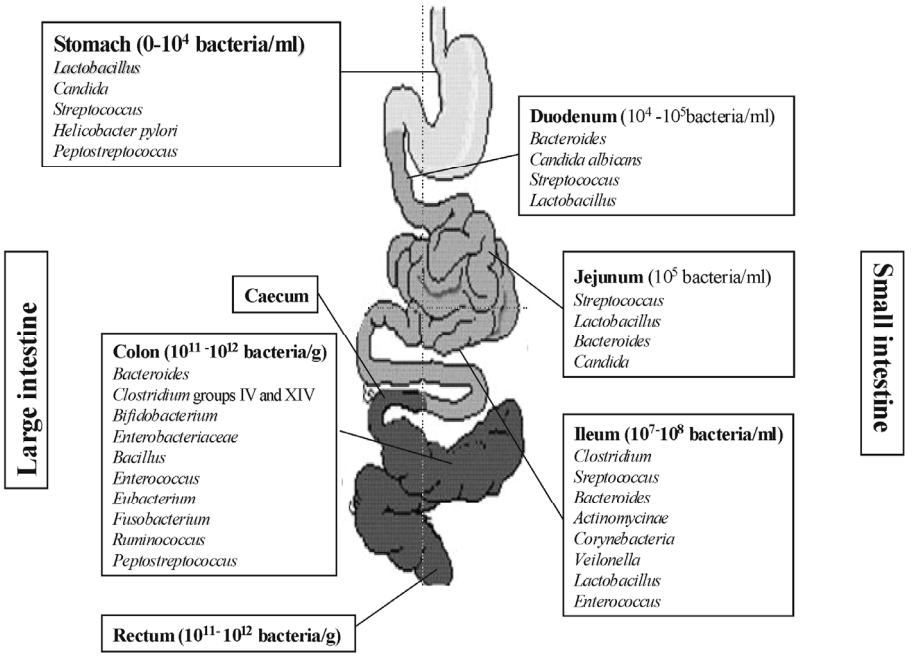


Figure 1. A bacterial distribution in human gastrointestinal tract (modified from Simon et al., 1984; Ouwehand et al., 2003).

2.1.2. The functions of the intestinal microbiota

The intestinal microbiota has important functions for the human host such as involvement in nutrition, immunomodulation and as a barrier against exogenous infections. Generally, these functions may be divided into three categories: structural, metabolic and protective (Guarner, 2006).

The gut microbiota is considered to represent a crucial line of defence against colonisation by exogenous or opportunistic bacteria that are present in the gut. This barrier effect involves several mechanisms, including displacement of pathogens by outcompeting them for the nutrients and epithelial-binding sites (Guarner, 2006), as well as production of antimicrobial factors such as lactic acid, hydrogen peroxide and bacteriocins, the latter inhibiting the growth of the invading bacteria (Shanahan, 2002). Its role in the development of a competent immune system cannot be neglected either (Guarner, 2006) and it is thought that the modulation of the immune system proceeds in a strain-dependent manner (Servin, 2004).

Gut bacteria also provide the metabolic functions involved in host nutrition. They have the ability to ferment non-digestible dietary substrates from the upper part of the gastrointestinal tract and endogenous mucus produced by the epithelia. The basic fermentative reaction in the human colon is hydrolysis of

polysaccharides, oligosaccharides, and disaccharides to their constituent sugars, resulting in an increased biomass. The fermentation of carbohydrates is the major source of energy in the colon for the bacterial growth, producing short chain fatty acids (SCFA) that can be absorbed by the host for structural completion of gut mucosa (McFarlane et al., 1995). A comparison of germ-free mice with conventional mice has shown that gut bacteria play a key role in the gut wall fortification by influencing the proliferation and differentiation of epithelial cells (Hooper et al., 2001).

The microbiota is also able to metabolise proteins and protein degradation products, sulfur containing compounds, as well as endogenous and exogenous glycoproteins (Gibson et al., 1995). In addition, gut bacteria are responsible for the production of vitamins such as K, B_{12}, biotin, folic acid, pantothenate, and in addition synthesis of amino acids from ammonia or urea (Hooper et al., 2002) as well as the inactivation of dietary carcinogens (Wollowski et al., 2001). However, few data are available on the metabolism of polyamines and biogenic amines by the intestinal microbiota (Arena et al., 2001; Matsumoto et al., 2007) or their specific roles in the aforementioned three functional categories of the microbiota in the host.

2.2. Lactic acid bacteria

LAB can be found in different nutrient-rich habitats, for example on the mucosal membranes of humans and animals, as well as on plants (Holzapfel et al., 2001). Bifidobacteria mainly originate from the gastrointestinal tract and breast milk of humans (Tannock, 1997; 2010). *Lactobacillus* spp. are ubiquitous and can be retrieved from dairy, grains, meat, fish, beer, wine, sauerkraut, sour dough, mash products, fruits, pickled vegetables, water, sewage, silage, and the mucosa of different human and animal cavities (mouth, intestine and vagina) (Salminen et al., 2004).

2.2.1. Taxonomy of lactic acid bacteria

LAB are a genetically distinct group of bacteria that share the same biochemical properties, e.g. gram-positive, non-sporulating rods or cocci, catalase negative, acid tolerant, lactic acid production as a result of carbohydrate fermentation and which prefer growth under anaerobic conditions but are quite aerotolerant (Wessels et al., 2004).

The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*. Important members of *Lactobacillaceae* are the genera *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Collins et al., 1987, 1993; Dicks et al., 1995; Adams and Moss, 2000; Felis et al., 2007). Its closest relative,

being grouped within the same family, is also the genus *Paralactobacillus* (Garrity et al., 2004). Nevertheless, after regrouping, the phylogenetically closest family appears to be the *Leuconostocacea* family (Hammes et al., 2003).

Genus *Bifidobacterium* has previously been considered in the same context as the genuine LAB while sharing some of their typical morphological and functional properties (Kandler and Lauer, 1974; Vaughan et al., 2002). However, Pourand et al. (1973) first postulated a relation of the genus *Bifidobacterium* to *Actinomycetaceae* group. Moreover, its place in the phylum *Actinobacteria,* order *Bifidobacteriales* and family *Bifidobacteriaceae* has been confirmed by analysis of 16S rRNA sequences (Woese, 1987; Garrity et al., 2004) and in addition, it has a specific pathway for sugar fermentation.

2.2.1.1. The genus *Lactobacillus*

Lactobacilli are gram-positive bacteria and vary in morphology from long, slender rods to short coccobacilli, which frequently form chains. They are fermentative, catalase-negative microaerophilic and chemo-organotrophic, producing lactic acid as the major product during the fermentation of carbohydrates (Axelsson, 1998). They have complex growth requirements supplied by carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins. A low pH (between 4.5 and 6.2) is stimulatory. The genus *Lactobacillus* is one of the largest, comprising more than 90 described species as well as some subspecies (Robinson, 2002; http://www.bacterio.cict.fr), and approximately 30% of them have been isolated from faecal sources (Table 2). The genus is very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. With regards to the DNA base composition of their genome, lactobacilli usually have GC content lower than 54%.

Lactobacilli can be subdivided into three groups according to the type of sugar fermentation they demonstrate: two genera of homofermenters '*Thermobacterium*', '*Streptobacterium*', and a third genus of heterofermenters '*Betabacterium*' (Table 1) (Kandler and Weiss, 1986; Pot et al., 1994; Hammes et al., 1995; Axelsson, 1998).

Group I – obligately homofermentative lactobacilli (OHOL) are able to convert the hexoses into lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway (Figure 2) while the pentoses and the gluconate are not fermented as OHOL lack phosphoketolase;

Group II – facultatively heterofermentative lactobacilli (FHEL) degrade the hexoses to lactic acid by the EMP pathway and the pentoses to lactic acid and ethanol/acetic acid via phosphoketolase. In addition, gluconate is often fermented (Figure 3);

Group III – obligately heterofermentative lactobacilli (OHEL) ferment the hexoses to lactic acid, carbon dioxide and ethanol (or acetic acid in the presence of an alternative electron acceptor). Furthermore, pentoses are converted to lactic and acetic acids (Figure 3).

Table 1. The division of the most common *Lactobacillus* ssp. (adapted from Botazzi, 1983; Axelsson, 1993).

Fermentation pathway	**Group I Obligately homo-fermentative**	**Group II Facultatively hetero-fermentative**	**Group III Obligately hetero-fermentative**
Growth at 45°C	+	+	+/–
Growth at 15°C	–(+)[#]	+(–)[**]	+(–)[**]
Hexose fermentation	+	+	+
Pentose fermentation	–	+	+
Fructose-diphosphate (FDP) aldolase	+	+	–
Phosphoketolase (PK)	–	+[*]	+
Gas from glucose	–	–	+
Gas from gluconate	–	+	+
NH_3 from arginine	–(+)[#]	–	+(–)[**]
Lactic acid modification	D-, L-, DL	D-, L-, DL	DL
	L. delbrueckii	*L. casei*	*L. brevis*
	L. acidophilus	*L. curvatus*	*L. buchneri*
	L. helveticus	*L. paracasei*	*L. fermentum*
	L. salivarius	*L. plantarum*	*L. reuteri*
		L. peptosus	*L. oris*
		L. sakei	*L. mucosae*
		L. rhamnosus	

[*] – inducible by pentose; [#] – mostly negative; [**] – mostly positive, with a few exceptions

The different lactobacilli species are able to use different pathways depending on the conditions and enzymatic capacity (Kandler et al., 1986; Axelsson, 1998). However, the division of lactobacilli into three main groups according to the type of fermentation is not in accordance with their phylogenetic classification as revealed by analysis of their 16S rDNA sequences (Axelsson, 1998). *Lactobacillus* spp. with widely diverse DNA base ratios can be grouped into three clusters: *L. delbrueckii* group, *L. casei-Pediococcus* group and the *Leuconostoc* group which also contains some lactobacilli (Collins, 1991). Nevertheless, the *L. delbrueckii* group was renamed as the *L. acidophilus* group, according to the more typical representative of this group (Schleiefer et al., 1995). Furthermore, based on DNA-DNA hybridization and other phylogenetic methods they were grouped into 8 major groups: *L. buchneri*, *L. delbrueckii*, *L. casei*, *L. plantarum*, *L. reuteri*, *L. sakei*, *L. salivarius* and *L. brevis* group (Salminen et al., 1998; Felis et al., 2005). The *L. casei* group comprises the recently revised species *L. zeae*, *L. casei*, *L. paracasei* and *L. rhamnosus* (Dicks et al., 1996).

2.2.1.2. The genus *Bifidobacterium*

The 29 species belonging to the genus *Bifidobacterium,* share phenotypical features typical to LAB, such as organic acid production. Bifidobacteria are gram-positive, catalase-negative, polymorphic branched rods that occur singly, in chains or clumps. They are non-spore-forming, non-motile, and non-filamentous. *Bifidobacterium* spp. are chemoorganotrophs, growing in an anaerobic environment, having a fermentative type of metabolism, and producing organic acids but not gas from a variety of carbohydrates. Their genome GC content varies from 42 to 67% (Sebald et al., 1965; Scardovi et al., 1986; Biavati and Mattarelli, 2001).

The optimum temperature for the isolation of the bifidobacteria species from a human host is 36–38°C. In contrast, that for the species from animals is slightly higher, at about 41–43°C and may even reach above 46°C. Furthermore, the initial optimum growth pH is between 6.5 and 7.0.

According to their phylogenetic analysis, the *Bifidobacterium* branch forms a coherent phylogenetic unit as their 16S rRNA sequences share over 93% similarity (Leblond-Bourget et al., 1996; Matsuki et al., 1998; Botaccini et al., 2010). For the phylogenetic differentiation between closely related bifidobacterial species some other genes including the elongation factor Tu (*tuf*) gene (Ventura et al., 2004), recombinase A (*recA*) gene (Ventura et al., 2003), ATP synthase subunit B (*atpD*) gene (Ventura et al., 2004), pyruvate kinase (Vaugien et al., 2002) and xylose-5-phosphate/fructose-6-phosphate (*xfp*) (Yin et al., 2005) have been introduced. Multiple PCR-based methods support and complement the 16S rRNA-based division of bifidobacterial species. A phylogenetic tree of *Bifidobacterium* spp. includes 5 major groups: *B. adolescentis*, *B. pullorum*, *B. asteroides*, *B. boum* and *B. pseudolongum*. Species *B. breve* and *B. longum* form a couple, as well as *B. minimum* and *B. psychroaerophilum*. However, *B. bifidum*, *B. magnum*, *B. scardovii* and *B. subtile* form distinct branches.

Nevertheless, there are taxonomic issues concerning the genus *Bifidobacterium* in particular regarding the species *B. longum-B. infantis*, recently united under the name *B. longum* and the recognition of their three biotypes (*infantis*, *longum* and *suis* types) using molecular methods (Sakata et al., 2002). In addition, another debated issue is the relationship between *B. animalis* and *B. lactis*, the latter recently reclassified as *B. animalis* subsp. *lactis.* So far there are 29 recognised *Bifidobacterium* species with 11 being isolated only from human host (Table 2).

2.2.2. Metabolism of lactic acid bacteria

2.2.2.1. Sugar metabolism of *Lactobacillus* spp.

Lactobacilli are able to ferment various carbohydrates and adapt to the various environmental conditions by changing their metabolism accordingly. Two major sugar fermentation pathways are recognised in lactobacilli: glycolysis (the Embden-Meyerhof pathway) and the pentose-phosphate pathway (pentosephosphoketolase or 6-phosphogluconate pathway) (Kandler, 1983).

Glycolysis is a sequence of reactions that converts glucose into pyruvate with the concomitant production of a relatively small amount of ATP (Figure 2). In these reactions, two substrate level phosphorylation reactions, involving phosphoglycerate kinase and pyruvate kinase, operate to yield energy. If the only end product of this pathway is lactic acid, the fermentation is referred to as homolactic fermentation. This pathway is used by all the lactic acid bacteria, with the exception of the leuconostocs and OHEL. Complete homolactic fermentation of glucose results in 2 moles of lactic acid and a net gain of 2 ATP per mole of glucose consumed.

The pentose-phosphate pathway is used in heterofermentative lactobacilli and the main difference from homolactic is the presence of enzyme phosphoketolase and lack of fructose-diphosphate aldolase (Figure 3). Glyceraldehyde-3-phosphate from the phosphoketolase reaction is metabolised to lactic acid similarly to glycolysis, and acetyl phosphate is converted to ethanol. Heterolactic fermentation of glucose gives 1 mole of each lactic acid, ethanol and CO_2 and 1 mole ATP per glucose consumed. In aerobic conditions, NADH can be oxidized by oxygen and acetate is produced from acetyl phosphate, yielding additional ATP from substrate level phosphorylation.

Facultatively and obligatory heterofermentative lactobacilli also ferment pentose via the lower half of the pentose-phosphate pathway, starting from ribulose-5-phosphate or xylulose-5-phosphate (Figure 3). The fermentation of pentoses results in production of equimolar amounts of lactic and acetic acids, while no CO_2 is formed (Axelsson, 1993). Metabolism of hexose is similar to that of glucose and begins from glucose-6-phosphate or fructose-6-phosphate (Figure 3).

Configuration of the lactic acid produced by lactobacilli depends on the presence of specific NAD^+-dependent lactate dehydrogenases (nLDH) and their respective activities in the cell. If both D- and L- lactic acids are formed, there are generally one D-nLDH and one L-nLDH present. In these cultures, generally L-lactate is the major form produced in early growth phase and D-lactic acid in the late stationary phase (Axelsson, 1993).

Lactobacilli may change their metabolism in response to various conditions and this can be attributed to an alternative pyruvate metabolism or the use of external electron acceptors. Pyruvate, intermediately formed in both pathways, may undergo several conversions (Figure 4).

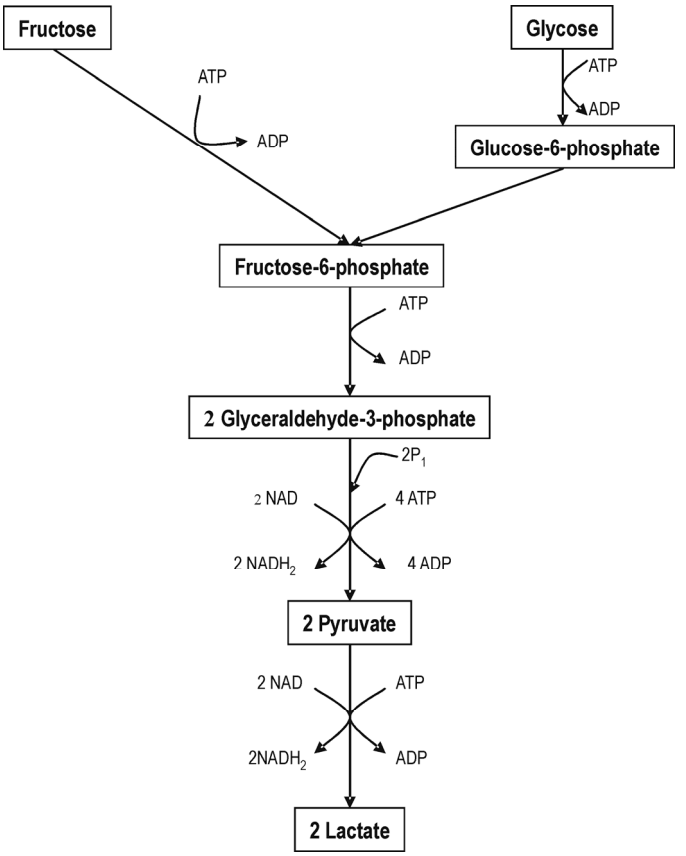


Figure 2. Homofermentative pathway of lactic acid bacteria, adapted from McDonald et al. (1991).

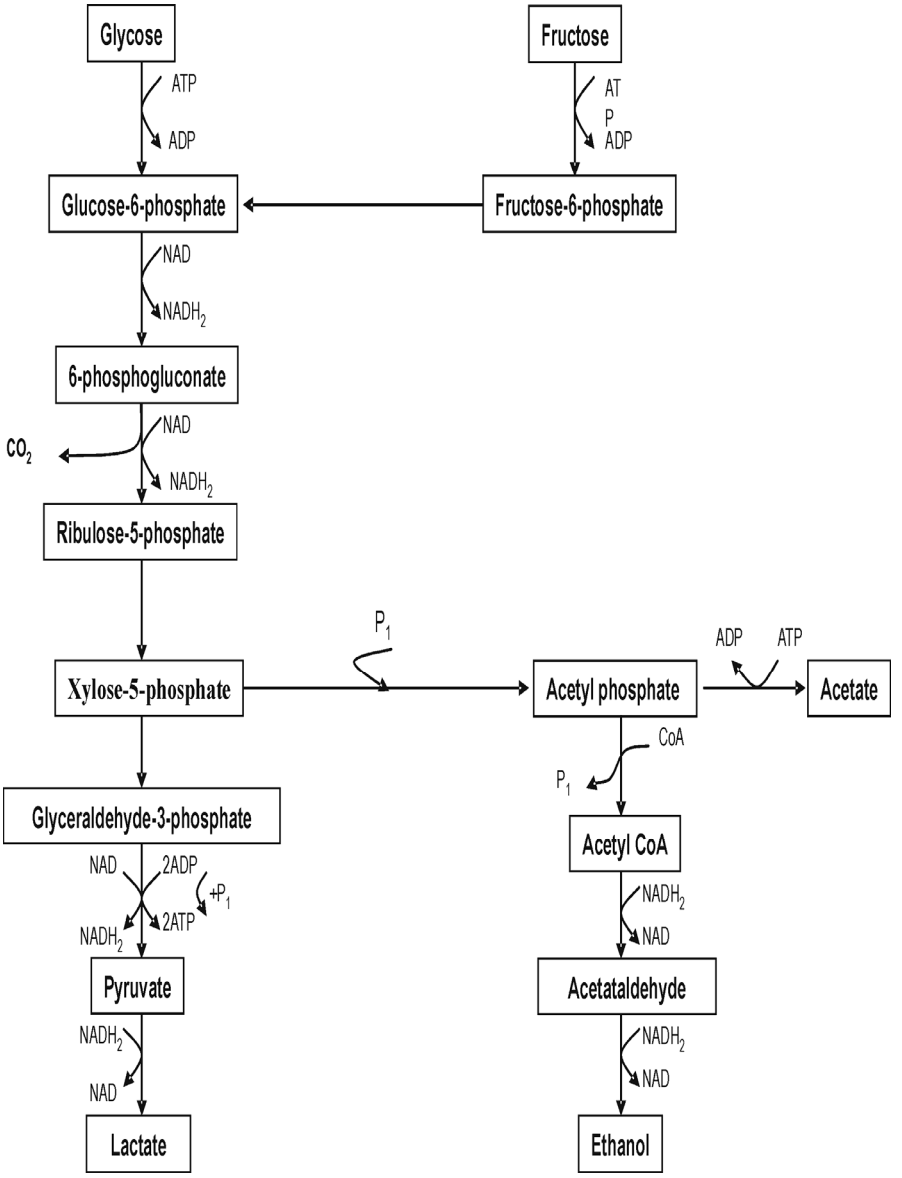


Figure 3. Heterofermentative pathway of lactic acid bacteria, adapted from McDonald et al. (1991).

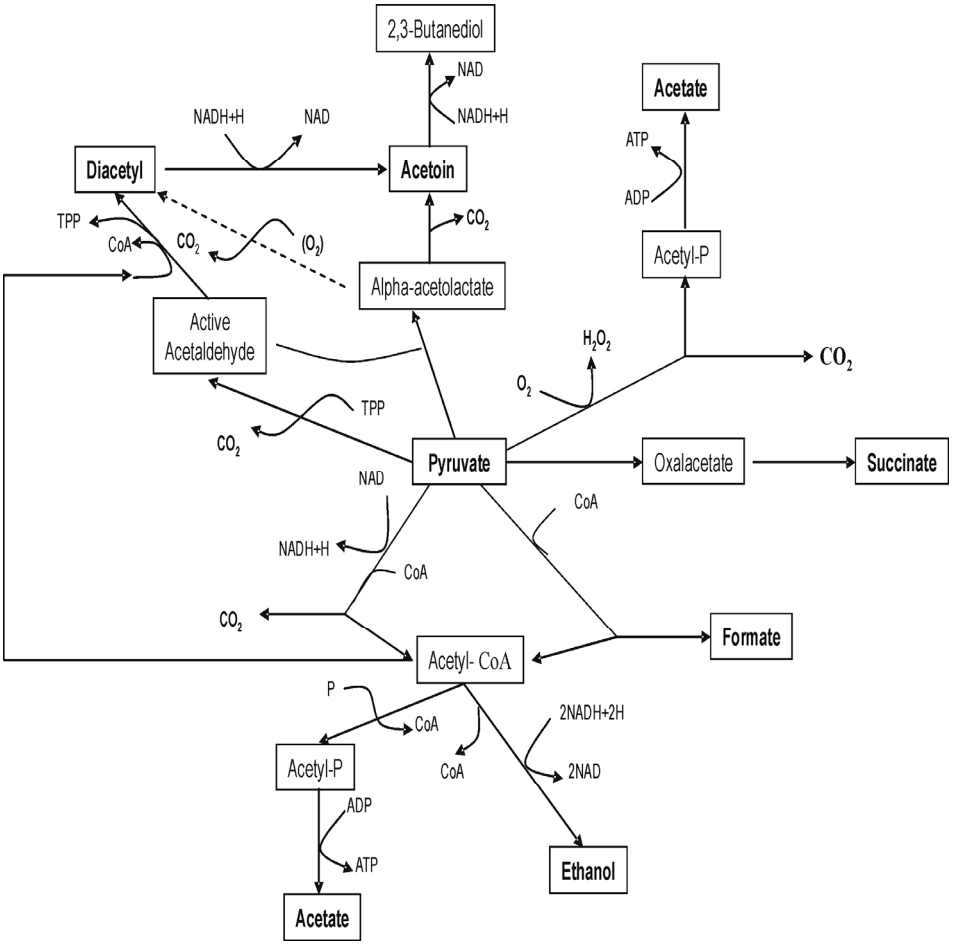


Figure 4. Pathways for the alternative fates of pyruvate, adapted from Axelsson (1998).

2.2.2.2. Sugar metabolism of *Bifidobacterium* spp.

Fructose-6-phosphate phosphoketolase (F6PPK) is the key enzyme of the hexose fermentation and F6PPK activity is one of the main phenotypic features used to identify bifidobacteria at the genus level. Lactic and acetic acids are produced as metabolic end products from hexose fermentation, as described by Scardovi and Trovatelli (1965). The second pathway involves the splitting of pyruvate by a phosphoroclastic enzyme to form formic acid and acetyl phosphate, a portion of which is subsequently reduced to form ethyl alcohol and so regenerate NAD (Figure 5) (Scardovi, 1986).

The proportions of the fermentation products vary considerably from one strain to another and even within the same species (De Vries et al., 1968). Small quantities of succinic acid are produced by some strains, and a small amount of CO_2 may be produced during the degradation of gluconate (Scardovi, 1986).

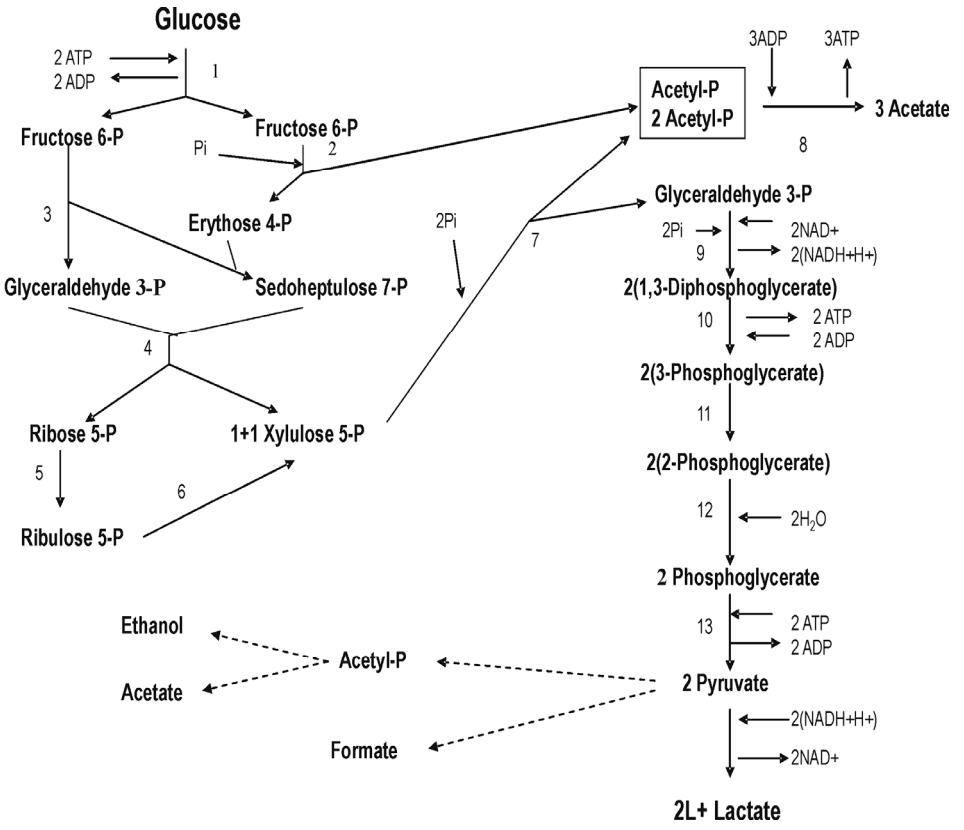


Figure 5. Metabolic pathway of *Bifidobacterium* spp. (Ballongue, 2004): 1– hexokinase and glucose-6-phosphate isomerase; 2 – fructose-6-phosphate phosphoketolase; 3 – transaldolase; 4 – transketolase; 5 – ribose-5-phosphate isomerase; 6 – ribulose-5-phosphate epimerase; 7 – xylose-5-phosphate phosphocetolase; 8 – acetate kinase; 9 – homofermentative pathway enzymes; 10 – (L+) lactate dehydrogenase; 11– phosphoroclastic enzyme; 12 – formate dehydrogenase; 13– alcohol dehydrogenase.

2.2.2.3. Poly- and biogenic amines

Polyamines are aliphatic molecules with amine groups distributed along their structure. Polyamines are known classically by the names of putrescine [1,4-butane diamine or tetramethylenediamine], spermidine [N-(3-aminopropyl)-1,4-butane diamine or aminopropyl-tetramethylenediamine] and spermine [N,N'-bis(3-aminopropyl)-1,4-butane diamine or diaminopropyltetramethylene-diamine]. They are present in all human cells (Larque et al., 2007). Other oligoamines and diamines such as histamine, tyramine and cadaverine also occur naturally, but they are not included usually with the general term of polyamines (Wallace, 2003) and are referred as biogenic amines.

Polyamines are water-soluble, with pK (dissociation constant) values of about 10 when fully protonated at body pH and are bound by a strong interaction with polyanionic macromolecules such as DNA and RNA. Only around 7–10% of the total cell content remains as free polyamines (Gugliucci, 2004; Moinard et al., 2005). The functions of polyamines depend on their electrical charges; their binding energy decrease from spermine to putrescine (spermine > spermidine > putrescine) (Yuan et al., 2001). The source of polyamines may be endogenous (intracellular *de novo* synthesis and interconversion pathways) or exogenous supplied in the diet.

Endogenously, polyamines can be synthesized from ornithine by a reaction catalysed by the enzyme ornithine-decarboxylase (ODS), which produces putrescine (Figure 6). Spermidine derives from putrescine after the addition of a propylamine group derived from the decarboxylated S-adenosylmethionine (SAM) by the action of spermidine synthase. Spermidine is similarly converted into spermine by the enzyme spermine synthase that adds a second propylamine group from SAM to the spermidine. Polyamine interconversion is performed by two coupled reactions: acetylation, mediated by the action of an acetyl coenzyme A: polyamine N'-acetyl transferase, and cleavage through the action of an enzyme, polyamine oxidase. However, there is an alternative pathway in which arginine is firstly decarboxylated to yield agmatine, a compound that breaks down to urea and putrescine (Figure 6). However, evidence for this alternative pathway in LAB is lacking (Moreno-Arribas et al., 2003).

The main source of exogenous polyamines is dietary (especially from cheese, fruit, meat, and some vegetables) and human milk (Kalaš et al., 2005; Larque et al., 2006). Human milk is very high in polyamines where they may account for its growth promotion properties and protective effects against allergies. The substantial amounts of spermine and spermidine of breast milk potentially modulate the maturation of the infant's intestines, enzyme activity and mucosal barrier functions (Wang et al., 1991; Buts et al., 1993; Capano et al., 1994; Deloyer et al., 2001). However, the larger amounts of putrescine can potentiate the effects of histamine by inhibiting the detoxifying enzymes diamine oxidase and hydroxymethyl transferase (Eerola et al., 1997; Guerrini et al., 2002).

The polyamines are involved in many physiological functions, including immunity. These chemical entities play also an important role in cell growth, proliferation and the synthesis of proteins and nucleic acids. Moreover, it has been reported that polyamines are involved in DNA transcription and RNA translation processes with their cellular storage in the cytosol and nucleus (Moinard et al., 2004). They are also involved in the repair of the extracellular matrix, cell adhesion and certain signaling processes. Polyamines depletion has been shown to inhibit cell proliferation and migration, or cause defective embryo development, whereas over-accumulation of polyamines induces apoptosis and cell transformation. In sufficient amounts the polyamines, particularly putrescine, are important in maintaining the healthy structure and function of

intestinal mucosa, a function which seems to also require vitamin D (Shinki et al., 1991). The exogenous polyamines derived from food are absorbed mainly in the upper parts of the intestine (Milovic, 2001; Bardocz et al., 1998).

Although the increase of polyamine synthesis is necessary for all of the tissue reparative processes, it is not known to what extent dietary polyamines intake may have a relevant role in the recovery of damaged tissue, especially the bowel and the liver, as well as their functional properties in aiding host resistance against infection. Nevertheless, a long term polyamine deficient diet results in the atrophy of the intestinal lining in both the small intestine and the colon of animals (Chamaillard et al., 1993; Loser et al., 1999).

The biogenic amines of microbial origin are formed from the decarboxylation of amino acids. An ability to form the biogenic amines has been described for several groups of microorganisms, mainly *Enterobacteriaceae*, *Pseudomonas* spp., enterococci and lactic acid bacteria (Halász et al., 1994; Lavizarri et al., 2010). The amino acids such as phenylalanine, lysine, histidine, tyrosine and methionine are the precursors to the following biogenic amines, respectively: phenethylamine, cadaverine, histamine, and tyramine. However, the conditions under which tyramine and histamine are produced by LAB have not been elucidated yet.

Biogenic amine-producing obligately heterofermentative lactobacilli such as *L. brevis*, *L. buchneri*, *L. divergens* and *L. hilgardii*, as well as the facultatively heterofermentative *L. carnis* and *L. curvatus*, have been isolated from meat and meat products. Edwards et al. (1983) showed that tyramine formation is restricted to some species of lactobacilli, particularly *L. divergens* and *L. carnis*. Some other lactobacilli may be responsible for the build-up of amines present in cheese. Histidine decarboxylase activity appeared to be species and strain specific. Some bacteria which are used as starter cultures in the dairy industry, such as *Streptococcus lactis* and *L. helveticus*, are identified as the histamine producers (Stratton et al., 1991). Other histamine-producing organisms including *L. buchneri*, *L. bulgaricus*, *L. plantarum*, *L. casei*, *L. acidophilus* and *L. arabinose* have also been shown to possess histidine decarboxylase activity (Stratton et al., 1991).

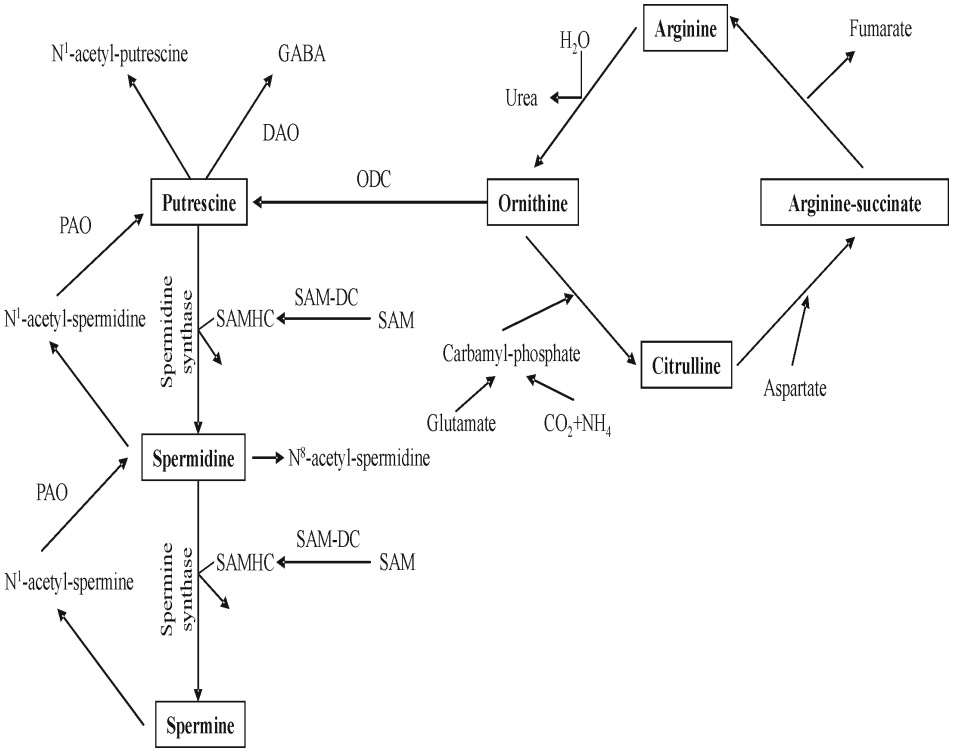


Figure 6. Synthesis and interconversion of polyamines. DAO, diamine oxydase; GABA, γ-aminobutyric acid; ODS, ornithine-decarboxylase; ADS, arginine-decarboxylase; PAO, polyamine-oxydase; SAM, S-adenosylmethionine; SAM-DC, S- adenosylmethionine decarboxylase; SAM-HC, S-adenosylmethionine homocysteamine (Larque et al., 2006).

2.3. The composition and functions of the intestinal lactic acid bacteria

2.3.1. The distribution of lactobacilli and bifidobacteria

Lactobacillus species are natural commensals of the gastrointestinal tract, oral cavity and female urogenital tract (Mikelsaar et al., 2004). Lactobacilli account no more than 1% of the faecal flora of adults (Franks et al., 1998; Sghir et al., 2000; Marteau et al., 2001; Zoetendal et al., 2006). In the stomach, at a pH of 3 (2.2–4.2), they are considered to be transient rather than resident species. In the large intestine, where the elementary sugars are rarely available, lactobacilli rely on the fermentation of the products from the other organisms able to degrade mucin and plant carbohydrates.

The microbiota of the small intestine increases from $< 10^4$ bacteria per ml of digesta in the duodenum up to 10^8 cells bacteria per g in the ileum. Total *Lacto-*

bacillus counts range from 3.98 x 10^3 to 3.16 x 10^{12} with a mean of 3.98 x 10^9 CFU per g of faeces. They are retrieved from 78% of faecal samples analysed (Gorbach et al., 1967; Drasar and Hill, 1974; Finegold et al., 1983; Tannock, 1995; Reuteri, 2001). The microbiota becomes more complex in the ileum resembling that of the large intestine, and also the relative proportion of lactobacilli drops (Walter, 2008).

Table 2. *Lactobacillus* spp. and *Bifidobacterium* spp. isolated from human faeces.

***Lactobacillus* spp.**	**Reference**	***Bifidobacterium* spp.**	**Reference**
L. acidophilus	Moro, 1900	*B. bifidum*	Tissier, 1900
L. crispatus	Finegold et al., 1977	*B. longum*	Sakata et al., 2002
L. gasseri	Lauer et al., 1980	*B. infantis*	Sakata et al., 2002
L. johnsonii	Fujisawa et al., 1992	*B. breve*	Reuter, 1963
L. jensenii	Carlsson et al., 1975	*B. adolescentis*	Reuter, 1963
L. amylovorus	Tannock, 1999	*B. angulatum*	Skardovii et al., 1974
L. delbrueckii	Finegold et al., 1974	*B. catenulatum*	Scardovii et al., 1974
L. helveticus	Finegold et al., 1977	*B. pseudocatenulatum*	Skardovii et al., 1979
L. salivarius	Moore et al., 1974	*B. dentium*	Skardovii et al., 1974
L. ruminis	Sharpe, 1977	*B. thermophilum*	Finegold et al., 1974
L. casei	Moore et al., 1974	*B. gallicum*	Lauer, 1990
L. paracasei	Dal Bello et al., 2006		
L. sakei	Heilig et al., 2002		
L. curvatus	Tannock, 1999		
L. mucosae	Decroos et al., 2005		
L. rhamnosus	Tannock, 1999		
L. plantarum	Finegold et al., 1974		
L. reuteri	Molin et al., 1993		
L. fermentum	Moore et al., 1974		
L. brevis	Tannock, 1999		
L.buchneri	Tannock, 1999		

Total *Bifidobacterium* counts range from 7.94 x 10^4 to 2.51 x 10^{13} CFU with a mean of 1.58 x 10^{10} cells per g of faeces. They can be detected from 74% of human stools (Finegold et al., 1983), comprising up to 10% of the total faecal microflora of adults (Langendijk et al., 1995; Franks et al., 1998; Sghir et al., 2000). They are more numerous in the infant gut where they form up to 91% of the total microbiota of breast-fed babies, and up to 75% in formula-fed infants (Harmsen et al., 2000). The most commonly detected lactobacilli and bifidobacteria species in the intestine are depicted in the Table 2.

2.3.1.1. The role of short-chain fatty acids in the intestinal tract

SCFA produced by the fermentation of carbohydrates are groups of organic acids with the chain lengths of up to 6 carbon atoms and include, acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, iso-caproic and caproic acids (Cummings et al., 1987; Axelsson, 1998).

Fermentation by LAB is characterised by the accumulation of organic acids and the accompanying reduction in pH level. The main fermentation products of LAB are acetic, lactic and succinic acids (Holdeman and Moore, 1975), the additional end products include formic, caproic, propionic, butyric, valeric acids and ethanol (Corsetti et al., 1998; Zalan et al., 2010). Succinic acid is created as a fermentation product of sugars with the carboxylate anion called succinate. Succinic acid is a dicarboxylic acid, produced in considerable amounts during fermentation of carbohydrates by FHEL and OHEL group. Succinate plays a biochemical role in the citric acid cycle and is capable of donating electrons to the electron transport chain leading to fumarate and ubiquinone. Also there are a few reports about the production of succinic acid from tartarate, malate and fumarate by some *Lactobacillus* strains (Radler, 1975; Whiting, 1975), which may play an important role in anti-oxidative processes. For instance, the production of succinate by *L. fermentum* ME-3 (DSM14241) seems to be one of the mechanisms for its antioxidative capacity (Mikelsaar and Zilmer, 2009). Succinic acid is a final product of the oxidation of putrescine in the small bowel of animals. 80% of putrescine is converted to succinate in fasting animals and may serve as a source of instantly metabolisible energy (Bardocz et al., 1998). Succinate lends to the fermented beverages such as wine and beer a common taste that is a combination of saltiness, bitterness and acidity (Whiting et al., 1975).

SCFA are readily absorbed from the human colon, and facilitate the absorption of salt and water by the colon, moreover, they can stimulate mucosal growth in the gut. Colonic epithelium derives 60–70% of its energy from SCFA. The content and type of organic acids produced during the fermentation process depend on the species of lactobacilli, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). However, there is limited information available about how the growth environment (microaerobic, anaerobic) facilitates the SCFA production. Moreover, there is always a necessity for evaluating the specific profile of SCFAs when assessing the functional properties of the putative probiotic candidates of *Lactobacillus* sp. strains. The application of lactobacilli against particular pathogens in the different intestinal tract compartments requires this kind of fundamental data.

2.3.2. Age related differences

The composition of the human microbiota has been closely associated with age and the major changes in the composition occur during early life. Moreover, there are clear changes in the intestinal microbiota content which occurs between neonates (0–30 days), infants (1–12 months), children (6–12 years), adults (20–64 years) and the elderly (<65 years) which have been studied (de Onis et al., 1996; Wenzel, 2007).

2.3.2.1. Neonates and children

The formation of microbiota in parallel with the development of the immune system starts from the very first days of life, and this may be influenced by several factors such as mode of birth, composition of maternal microbiota, diet, environmental conditions and use of antibiotics (Wold et al., 1998; Grönlund et al., 1999, Strannegard et al., 2000; Harmsen et al., 2000). At birth, the intestine is sterile but within a few hours, bacteria start to appear in the faeces. As a result of the intestinal environment showing a positive oxidation/reduction potential at birth, the gastrointestinal tract is first colonised by facultative anaerobes, such as *Escherichia coli*, *Streptococcus* spp., *Staphylococcus* spp. and *Enterococcus* spp. Gradually, the consumption of oxygen by these bacteria changes the intestinal environment into a more-reduced one, permitting the subsequent growth of strict anaerobes, such as *Bifidobacterium*, *Bacteroides* spp. and *Clostridium* spp. (Bezirtzoglou, 1997). The bacteria colonising the infant gut during the first days of life originate mainly from the mother and the environment.

One of the first major determinants of the gut microbiota is the mode of delivery. A vaginally-born infant is exposed to bacteria from the mother (vagina and faeces) and the environment. Furthermore, the gut microbiota of infants delivered by caesarean section has been reported to differ from that of infants delivered vaginally, both in the timing of colonisation and in composition (Mändar et al., 1996; Gronlund et al., 1999; Penders et al., 2006). The caesarean-born infants are initially colonised by the strains from their mothers, the hospital environment and health care workers (Bezirtzoglou, 1997; Gronlund et al., 1999; Penders et al., 2008).

The second important factor that can influence the composition of the intestinal microbiota in infancy is the type of feeding used (Heavey et al., 1999; Penders et al., 2005). Breast-fed infants traditionally have a colonic population that are dominated by bifidobacteria and lactic acid bacteria, with very few bacteroides, clostridia and coliforms. More diversity occurs in the microbiota of the formula-fed infants that tend to contain large numbers of bacteroides, clostridia and enteric bacteria. However, during weaning, substantial temporal changes occur and the microbiota of young children only stabilises by the end of the second year. Consequently, at the age of 5 it resembles that of an adult in terms of composition (Rotimi and Duerden, 1981; Adlerberth et al., 1996) and metabolism (Midtvedt and Midvedt, 1992). The prevalence of bifidobacteria

and its metabolites decreases due to the changes of food in young children. Since the mid-1990s several research teams have been involved in elucidation of the impact of environmental factors on the well being of children, adults and elderly people (Adlerberth et al., 1991; Bennet et al., 1991; Sepp, 1998). Thus, country-specific differences have been postulated.

2.3.2.2. Adults and elderly people

Due to the altered physiological characteristics of elderly people, including a decreased intestinal motility, reduced secretion of gastric acid, and change of dietary habits and lifestyle, the microbiota of elderly persons differs from that of younger adults, despite expressing significant individual variations (Mitsuoka, 1992; Silvi et al., 2003; Hebuterne, 2003; Woodmansey et al., 2004; Claesson et al., 2010). The mechanisms underlying the observed age-dependent differences in microbiota composition are unknown. In elderly persons, it is common to have a reduction in the numbers and diversity of *Bacteroides* spp. and *Bifidobacterium* spp., and also a reduced production of SCFA and amylolytic activity. In addition, increased numbers of facultative anaerobes, fusobacteria, clostridia, eubacteria and fungi have been reported in elderly people (Gorbach et al., 1967; Woodmansey, 2007). Moreover, the frequency of isolation of *Clostridium difficile* is higher in the elderly. While this is partly a result of factors such as hospitalisation and nursing home care, clostridia in general have been found to occur in significantly higher numbers in healthy elderly volunteers compared with younger subjects (Ljungberg et al., 1990; Hopkins et al., 2001). Although there is a large disagreement in the findings between studies, probably due to individual differences and the use of different methodologies, the abundance and diversity of bifidobacteria is consistently reported to be decreased in elderly individuals (Mueller et al., 2006; Woodmansey, 2007).

In contrast an increased prevalence in the numbers of *Lactobacillus* spp. during aging has been described (Mitsuoka, 1992; Tiihonen, 2008), and often described as country-specific (Silvi, 2003; Mueller, 2006). Recently, some studies have reported an association between the presence of intestinal lactobacilli and an impact on metabolism and energy uptake in the host (Cani et al., 2009). Although, there are some comparative studies of *Lactobacillus* spp. diversity between adults and elderly groups, these are carried out using different methods, such as bacteriological methods (Woodmansey et al., 2004; Vassos, 2007) or denaturing gradient gel electrophoresis (DGGE) with *Lactobacillus* genus-specific primers (Song et al., 2000; Nielsen et al., 2003; Cagno et al., 2009).

2.3.3. Environmental differences

A change in microbial ecology prompted by Western diets, and/or differences in microecology between individuals living in these societies, may function as the "environmental" factors.

2.3.3.1. Geographical and social differences

In various geographical regions differences of diet emerge, usually playing an important role in the composition of intestinal microbiota (Finegold et al., 1974; Salminen et al., 1995). More anaerobic bacteria are found in the gut microbiota of Swedish children and American adults who are consuming a Western diet compared to that of Japanese, Chinese and Estonian subjects. However, Japanese who are living in Western countries for many years obtain a new type of gut microbiota (Reddy et al., 1973; Finegold et al., 1974; Sepp et al., 1997; 2006). On the other hand, in modern developed Western countries the environmental factors such as the higher hygienic life style may cause shifts in intestinal microbiota (Dunder et al., 2001; Alm et al., 2002). There are several studies on diversity of the human intestinal microbiota between different countries (Lay et al., 2005; Mueller et al., 2006; Fallani et al., 2010). Moreover, children living in countries with a lower level of industrialisation have a significantly higher number of LAB such as enterococci and lactobacilli (Bennet et al., 1991; Sepp et al., 1997).

It has been shown that there is a greater predominance of enterobacteria in Estonian neonates during their first week of life in comparison to Finnish newborns (Mikelsaar, 1992). However, the increased industrialisation in late 1990s could have had an impact on the changes of gut microbiota in former socialist countries after regaining independence. This may be related to the changes in lifestyle and a more strict level of hygiene (Sepp et al., 1997, 2006).

2.3.4. Gut microbiota in diseases

2.3.4.1. Allergy

Potential explanations for the increased prevalence of eczema and other atopic diseases include reduced exposure to the microbial agents (so-called "hygiene hypothesis") (Strachan, 1989) and/or changes in the gut microbiota in early life (Marticardi et al., 2001). The development of allergic disease could be associated with an imbalance of gut microbial ecosystem. Normally, due to colonisation resistance, the major groups of anaerobes such as bifidobacteria, eubacteria, bacteroides and peptostreptococci suppress the potentially pathogenic micro-organisms such as aerobes and clostridia.

A few prospective studies have examined the relationship between the composition of the gut microbiota in early life and the development of atopy (Kalliomaki et al., 2001; Adlerberth et al., 2007; Penders et al., 2007; Wang et

al., 2007). A culture-based study of stool samples of 324 European neonates followed from birth to the age of 18 months found that neither time of gut colonisation with 11 bacterial groups nor ratio of strict anaerobes to facultative anaerobes was associated with eczema or food allergy (Adlerberth et al., 2007). In contrast, a more advanced study of 957 Dutch infants showed that the presence of *C. difficile* in stool samples at the age of 1 month (assessed by quantitative real-time PCR) was associated with an increased risk of eczema, recurrent wheeze, and allergic sensitization at the age of 2 (Penders et al., 2007). Moreover, in that study, early colonisation with *E. coli* was associated with eczema by parental report but not with objectively diagnosed eczema.

There is an increasing prevalence of allergic multifactorial diseases in the industrialised countries. Moreover, some studies of children in the former Soviet countries have indicated a lower prevalence of allergic disease than in countries with a market economy (Björkstén, 1994; Riikjärv et al*.,* 1995). According to several studies, bifidobacteria are less commonly detected in children and adults with allergic disease than in healthy persons (Björksten et al., 2001; Kalliomäki et al., 2001; Watanabe et al., 2001). In addition, children who developed allergy were significantly less colonised with *L. rhamnosus, L. casei, L. paracasei*, *B. adolescentis* and *C. difficile* during their first 2 months of life. Nevertheless, the infants colonised with several *Bifidobacterium* species had been exposed to the higher amounts of endotoxin and grew up in the larger families than infants harbouring a few species (Sjogren et al., 2009).

The deprivation of microbial abundance has been proposed as a reason for the development of allergy in genetically predisposed infants (Björkstén et al., 2001). However, molecular studies starting from infancy are needed to quantify LAB numbers and composition, comparing the different populations with and without developing allergy. It has not been established yet, how long the changed during infancy composition of intestinal microbiota is kept specific for allergy.

2.3.4.2. Metabolic syndrome

An obesity epidemic has spread all over the world during the past 30–40 years. Persons becoming clinically obese have a higher risk of developing dysmetabolism. This is characterised by ectopic fat accumulation resulting in increased triglyceride content and reduced HDL-cholesterol in the blood, arterial hypertension and type 2 diabetes. Today, this phenotype is designated metabolic syndrome (Beck-Nielsen et al., 2010). The term "metabolic" refers to the biochemical processes involved in the body's normal function (Kalliomäki et al., 2008).

It is suggested that obesity is associated with the genome of the human micorbiota which encodes its metabolic capacities (Bäckhed et al., 2004; Gill et al., 2006; Vrieze et al., 2010). Studies have shown that the diabetics have a different composition of bacteria living in their digestive tracts (Larsen et al., 2010). Reduced glucose tolerance is a key issue in diabetes and it has been

shown that the intestinal microbiota of persons with diabetes consist of lower levels of bacteria from the phylum *Firmicutes* and class *Clostridia*, and higher amounts of bacteria from the phylum *Bacteroidetes* (Ley et al., 2005). The researchers have also found a positive correlation between the ratios of *Bacteroidetes* to *Firmicutes* and plasma glucose concentration (Larsen et al., 2010). *Firmicutes* enable hydrolysis of indigestible polysaccharides to easily absorbable monosaccharides and activation of lipoprotein lipase by direct action on the villous epithelium. In addition, the ratio of the *Bacteroides-Prevotella* group to the *Clostridium coccoides-Escherichia rectale* group has been correlated positively and significantly with plasma glucose concentration. Furthermore, a reduction in *Firmicutes* phyla such as *Clostridium histolyticum* and *Eubacterium rectale-Clostridium coccoides* has been significantly correlated with a reduction in weight and body mass index score (Nadal et al., 2009). Moreover, in a study of elderly people with high frailty scores, a significant decline in numbers of lactobacilli-enterococci has been shown (van Tongeren et al., 2005).

Furthermore, during pregnancy the microbiota composition has shown to be related to body weight, weight gain and metabolic biomarkers, which might be of relevance to the management of the health of women and infants. The comparison of over-weight to normal-weight pregnant women showed that the former had reduced numbers of *Bifidobacterium* spp. and *Bacteroides* spp. and increased numbers of *Staphylococcus* spp., *Enterobacteriaceae* and *Escherichia coli* (Santacruz et al., 2009). Moreover, the *E. coli* numbers were higher in women with excessive weight gain compared to women with normal weight gain during pregnancy, whilst *Bifidobacterium* and *Akkermansia muciniphila* showed an opposite trend. In addition, increased counts of total bacteria and *Staphylococcus* spp. numbers have been related to the increased plasma cholesterol levels (Santacruz et al., 2009). These recent studies suggest that *Bifidobacterium* spp. and *Bacteroides* spp. may protect against the development of obesity.

It has also been shown that in infancy, the bifidobacterial numbers were higher and the number of *Staphylococcus* spp. were lower in faecal samples from children with normal weight compared to overweight children (Kalliomaki et al., 2008).

2.4. Methods for studying lactic acid bacteria

Classical techniques for analysing LAB in intestinal microbiota include culture-dependent and culture-independent approaches.

2.4.1. Bacteriological and biochemical methods for identification of lactic acid bacteria

Traditionally, analysis of the composition of the gut microbiota relied on the use of bacteriological methods such as cultivation on specific medium, microscopy and identification (O'Sullivan, 1999; Finegold et al., 1983; Moore and Holdeman, 1974). After isolation of colonies it is necessary to confirm the genus identity and characterise on the species (or strain) level. For *Lactobacillus* spp. this characterisation requires a battery of classical morphological and biochemical tests described in the Bergy's Manual of Systematic Bacteriology (Bergey, 1986). In addition, a bacterial count in the original sample is determined by multiplying the number of colonies that develop with the degree of dilution.

Phenotypic methods have been the most commonly used for the identification of LAB. The phenotypic differentiation between species of lactobacilli relie on the carbohydrate fermentation pattern, configuration of lactic acid (L-, D-, LD-isomers) and hydrolysis of arginine, requirements for the growth at certain temperatures (Orla-Jensen, 1919; Sharpe, 1981; Botazzy, 1983). There is a detection kit developed by bioMerieux (France) for the identification of lactobacilli. The system is called an Analytical Profile Index and is based on the fermentation pattern of 50 carbohydrates by *Lactobacillus* (isolates) (API 50 CHL). The kit is mainly used for the identification to species level, and its precision can be greatly improved by computerized application of Bayes's theorem (Cox and Thomsen, 1990). The great advantage of cultivation is that the isolates can be recovered and further studied for their ability to utilise the different substances and also other physiological parameters, including their antibiotic susceptibility pattern.

However, cultivation suffers from several drawbacks. Firstly, only a small proportion (approximately 40%) of the fastidious bacterial community residing within intestinal tract can be cultivated with the approaches currently available (Tannock et al., 2000; Eckburg et al., 2005). Secondly, the labour intensity and finances necessary for classical bacteriology are remarkably high thus limiting the effectiveness for analysing a large number of individuals.

2.4.2. Gas-liquid chromatography for studying metabolic activity of lactic acid bacteria

Gas chromatography (GC) has been one of the most versatile and widely applicable techniques leading the field of analytical chemistry over the last 40 years. GC is a technique used to separate volatile and semi-volatile organic compounds in a mixture. In gas-liquid chromatography (GLC), the compounds move through a heated column in a mobile carrier phase (e.g. helium gas) and are separated by the different rates at which they move through the stationary phase (an inert support material coated with a liquid resin). At the end of the

column there is a detector that is sensitive to changes in temperature and in the flow of electrical current. As the compounds leave the column, the change in current is amplified and recorded on a strip chart recorder. The height of each peak produced is proportional to the amount of each compound present. Identification of a peak is accomplished by comparing it with the retention time of standards or known solution of compounds.

2.4.2.1. Detection of organic acids

GLC has been used for almost 35 years as a method for the detection of organic acids produced by bacteria. Bacteria produce characteristic metabolic products, including volatile SCFA (formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids), alcohols and non-volatile organic acids (lactic, fumaric and succinic acids) that are distinctive for the various groups or species. GLC analysis of metabolic end-products serves as a tool for classification of microorganisms to the genus level (Holdeman et al., 1972).

GLC of fatty acid methyl esters is widely used (Holdeman et al., 1972). Several methodologies using GLC have been elaborated. For example, Sutter et al. (1972) proposed to directly inject the acidificated culture medium in the column without prior treatment of the samples, despite the limited success to characterise anaerobic bacteria shown previously (Wiggins et al., 1985; Socolowsky et al., 1990). This method allowed rapid determination of volatile acids but did not succeed in identifying non-volatile acids. In addidtion, ghosting and tailing peaks appeared after a few injections, which promoted loss of sensitivity (Socolowsky et al., 1990). However, Lambert and Moss (1972) analysed non-volatile and volatile organic acids after obtaining their butyl esters. However, this method was relatively time-consuming and included solvent extraction and evaporation steps which could lead to the considerable loss of free acids in sample. In the same way, Holdeman et al. (1977) extracted acids and derivitized these to methyl esters, and also Carlier and Sellier (1987) studied methylated and butylated SCFA. Consequently, using GC and mass spectrometry, they succeeded to identify some SCFA, which are exclusive determinants for the identification of some particular strains of *Fusobacterium* spp. and *Clostridium* spp.

All species of lactobacilli produce primarily lactic acid; some also produce ethanol or detectable amounts of acetic, succinic, or formic acids. However, there are only limited data available regarding the variation of the fermentative reactions in the different oxidative environments (microaerobic, anaerobic) in which the cultures are grown.

2.4.2.2. Detection of poly- and biogenic amines

Although historically amines present difficulties in GLC analysis, it is possible to detect them if they are derivatized to become volatile prior to investigation (Kataoka, 1996). Different methods can be employed to derivatize amines such as acylation, silylation, and carbamate formation. For derivatization of primary and secondary amines the acylation reaction with the N-methyl-bis-trifluoroacetamide (MBTFA) has been used (Kataoka, 1996). Advantages of using acylation as a derivatization technique for amines are that the reaction occurs readily in mild conditions, and not only amines but other active substituents like phenols, thiols, and hydroxyls can become derivatized (Kataoka, 1996).

For silylation reactions with amines two reagents such as N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and N-methyl-N-(tert-butyldimethylsilyl) rifluoroacetamide (MTBSTFA) can be used (Drouett-Coassolo et al., 1989). BSTFA derivatizes hydroxyl and carboxyl groups, which is most effective under the anhydrous reaction conditions. Therefore, the aqueous samples are not recommended for such reaction. However, the primary amines are more readily derivatized than secondary amines. The advantage of using MTBSTFA is that generates derivatives that are orders of magnitude more stable for hydrolysis (Kataoka, 1996).

The most practical methods used to derivatize amines for GLC is through carbatate formation. The derivatisation of amines by alkylchloroformates in an aqueous enviromnent was extensively studied in the early 1980s (Ahnfelt et al., 1980). Consequently, primary, secondary and tertiary amines are derivatized with alkyl chloroformate reagents (Husek et al., 1998) and organic solvents, buffered aqueous solution or a two-phase system of organic solvent and water could be used. Also, chloroformates have been widely used to convert amines into carbamates in biological materials (Ugland et al., 1997).

2.4.3. Molecular tools for analysing of lactic acid bacteria

Within the last decade the microbial ecology of gastro-intestinal (GI) tract has been revised due to the development of wide variety of molecular techniques. It has been estimated that the microbial world harboured within us is significantly larger than expected. Modern molecular methods mainly based on ribosomal RNA (rRNA) and the encoded genes have revealed many intestinal bacterial species not previously described or cultivated (Suau et al., 1999; Zoetendal et al., 2001; Eckburg et al., 2005; van der Waaij et al., 2005; Frank et al., 2007).

2.4.3.1. Qualitative molecular methods

The 16S rRNA sequence analysis

The 16S rRNA has been the most widely employed molecule to develop the phylogeny of prokaryotes. The analysis of rRNA sequences has revealed sig-

nature sequences, short stretches of rRNA, that are unique to a certain group or groups of microorganisms enabling the phylogenetic identification of bacteria and detection of evolutionary relationships between species (Olsen et al., 1986; Amman et al., 1995).

The 16S rRNA is characterised by genetic stability in its domain structure with conserved and variable regions, and its high copy number (Woese, 1987). A comparison of sequences of different bacterial 16S rRNAs shows that the molecule contains segments with a different degree of variability. Currently, more than twenty thousands 16S rRNA sequences are available (Maidak et al., 2001). This allows the design of nucleotide-probes and primer sets that hybridize with a particular sequence in the 16S rRNA molecule.

The phylogenetic framework provided by the comparison of 16S rRNA gene sequences offers a conceptual approach to microbial identification and taxonomy. 16S rRNA gene sequences contain regions conserved across all bacterial species interspersed with regions (V1–V9) in which the nucleotide base sequences are variable among bacterial types (Stackebrandt and Goebel, 1994). Sometimes, the variable regions are highly species-specific. Comparison of 16S rDNA sequences can therefore be used in the identification of bacterial species and consequently, in the analysis of bacterial communities (Raskin et al., 1997). Universal or group-specific primers can be used in PCR to amplify 16S rDNA from bacterial cells in biological samples. The amplified 16S rDNA sequences can be cloned, screened and sequenced. Alignment of the sequence with those stored in databanks permits the recognition of the species represented in the habitat and detects those that cannot be cultivated using conventional bacteriological techniques.

From an rRNA sequence obtained using a PCR-cloning and sequencing approach, it is possible to determine the abundance of the corresponding micro-organism, its cell morphology and its spatial distribution using in situ hybridization.

However, there are some deficiencies in the use of 16S rDNA for studies of biodiversity. One is that in terms of size, the genes of the 16S molecule are extremely constant with a total variation of about 200 bp for a mean length of 1550 bp (Rainey et al., 1994) and therefore different genes cannot be easily separated by size. Additionaly, the 16S sequence, in spite of having hyper-variable and extremely informative regions for close relationships, is often not divergent enough to give good separation in close relationships, e.g. species of the same genus (Normand et al., 1996). Among the strains of the genus *Lacto-bacillus* and *Bifidobacterium,* the rRNA sequence is highly conserved (Leblond-Bourget et al., 1996) and may not be sensitive enough for the desired level of comparative analysis that is likely to be needed for the selection of worthwhile strains.

Currently, the complete genomes of more than 20 *Lactobacillus* and 9 *Bifi-dobacterium* strains are available. The genomes of lactobacilli and bifido-

bacteria have sizes varying from 1.8 to 3.3 Mb and from 2.0–2.8 Mb, respectively (Kant et al., 2010; Bottacini et al., 2010).

16S–23S rRNA intergenic spacer region analysis

In prokaryotes, the three genes coding for rRNA (16S, 23S and 5S rRNA) are separated by spacer region (Figure 7). Most known prokaryotes have genes coding for the different RNAs of an assembled ribosome organized into an operon as the functional transcription unit. The number of these operons for a given species largely depends on its growth rates and can range from 1 to 11 (Kostman et al., 1992; Gatcia-Martinez et al., 1996). Moreover, the spacer regions within a single strain may differ in respect of their sequence length.

The intergenic/internal transcribed spacer (ITS), also known as the intergenic spacer region (ISR) (Jensen et al., 1993; Toth et al., 2001; Shaver et al., 2002) between 16S and 23S rDNA genes, includes both conserved and highly variable sequence motifs, such as tRNA genes, ribonuclease III (RIII) enzymes and anti-terminators (*box A*) (Figure 7) (Garcia-Martinez et al., 1999; Abd-El Haleem et al., 2002). The size of the spacer may vary considerably for the different species, and even among the different operons within a single cell, in the case of multiple operons (Condon et al., 1995).

This region is extremely variable in size and sequence even within a closely related taxonomic group (Gürtler and Stanisich, 1996). Size patterns can be used to characterise different communities of bacteria, and the widely divergent sequence allows the detection of species-like units very precisely using PCR and oligo-probe hybridization (Jensen et al., 1993).

To complement the rRNA sequence approach, analysis of another molecule, which is not as conserved as 16S RNA but still retains the characteristics of a meaningful phylogenetic marker, is required. Two important criteria for such a molecule are that it is universally present in bacteria and it has high sequence conservation, where sequence changes are less influenced by temporary environmental changes. The region between the 16S and 23S rRNA genes, termed ITS, has been used for more detailed analysis of bifidobacteria and lactobacilli (Leblond-Bourget et al., 1996; Jacobsen et al., 1999). This molecule is universally present in bacteria, but can exhibit very low sequence conservation (Barry et al., 1991), thus limiting its accuracy as a phylogenetic marker. In addition, the ITS regions within the same bacterial strain can exhibit heterogeneity (Garcia-Martinez et al., 1996; Christensen et al., 2000). However, the molecule is technically easy to obtain, as PCR can be used to amplify it directly from colonies using primers directed at universally conserved regions within the bordering 16S and 23S rRNA genes. Leblond-Bourget et al. (1996) have evaluated the sequence analysis of this molecule to further characterise bifidobacteria and found it much more sensitive than the rRNA analysis. Furthermore, Tannock et al. (1999) demonstrated its usefulness for the identification of intestinal *Lactobacillus* spp.

Reverse transcriptase was used to generate DNA from rRNA, and this DNA was then sequenced. It is now possible to sequence 16S or 23S rDNA molecules by direct PCR, and this method has generated a large sequence database. Although the species-specific sequences are located in the first half of the 16S rRNA gene (V1–V3 region), identification is more accurate if the whole genome is sequenced (Stackebrandt et al., 1994). This requires the sequencing of about 1.5 kb of DNA.

Tannock et al. (1999) showed that comparison of the 16S–23S spacer region sequences of lactobacilli can be used in practical situations for the identification of strains. The 16S–23S spacer sequences of lactobacilli are small, only about 200 bp in length. These short sequences are easy to sequence on both strands and provide reliable information for comparative work.

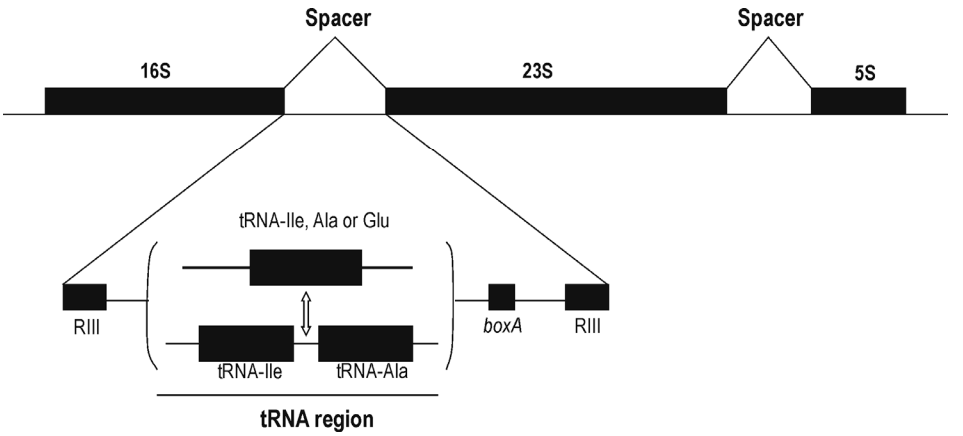


Figure 7. Structure of ITS region. (5S, 16S, 23S rDNA-genes, includes such as tRNA genes, ribonuclease III (RIII) enzymes, *box* A-anti-terminators; adapted from Garcia-Martinaz et al., 1999).

Denaturing Gradient Gel Electrophoresis and Temperature Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are among the most common fingerprinting approaches to assess the structure of complex biological systems. The general principle of DGGE/TGGE is the separation of individual rRNA genes based on differences in chemical stability or melting temperature of genes. One end of the fragment is held together with a so-called GC-clamp, which is an approximately 30 to 50 bp long C+G-rich sequence that is attached to the 5'-end of one of the primers. These methods separate multitemplate PCR products as bands on gels according to GC content, dependent on melting behaviors of the amplicons as they migrate thorough the gels. Polyacrylamide gels consisting of a linear denaturing gradient, formed by urea and formamide are employed for DGGE, whereas a linear

temperature gradient is used during TGGE. The resulting banding patterns on gels can be compared visually on the gel or analysed by cluster analysis (Muyzer et al., 1993). The advantage of this approach is that bands of interest can be excised and sequenced to obtain information about the species that they represent. The limitation of DGGE/TGGE is that heterologous sequences may migrate similarly, and thus bands at the same position in the gel are not neccessarily phylogenetically related (Muyzer et al., 1993; Muyzer et al., 1998).

PCR-DGGE/TGGE may be used for whole community analysis, or for the investigation of specific populations or groups within the sample. This method has been useful for analysing human faecal microbiota in health and disease (Walter et al., 2001; Zortendal et al., 2001; Favier et al., 2002), monitoring dynamic changes in mixed bacterial populations over time (Zoetendal et al., 1998; Heilig et al., 2002; Favier et al., 2002), assessing the effect of antibiotic therapy, probiotics and prebiotics on the faecal microbiota of patients and volunteers (Donskey et al., 2003). Moreover, it has been used to assess the microbial composition in mouth (Maukonen et al., 2008), stomach (Monstein et al., 2000) and breast milk (Delgado et al., 2008). The PCR-DGGE of 16S rRNA (or DNA) and 16S–23S ITS-region rRNA is widely used for selective monitoring of lactic acid bacteria and bifidobacteria populations (Walter et al., 2001; Heilig et al., 2002; Murray et al., 2005; Vaughan et al., 2005). Satokari et al. (2001) studied the bifidobacteria microbiota in adults using DGGE, and showed that *B. adolescentis* is the most common species found in the faeces of human adults.

Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) employs an alternative fields of electrophoresis to allow the separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes (*ApaI*, *SmaI*, *NotI*, *SfiI*, *XbaI*, *SacII*) with increasing pulse times throughout the run. The resulting fingerprint profiles can be explored for culture identification (O'Sullivan et al., 1999; Holzapfel et al., 2001). PFGE has been widely applied in the analysis of bacterial genomes. This tehnique has shown excellent discriminatory power in comparison to other typing methods in the differentiation of lactobacilli strains (Kimura et al., 1997). Strain typing has been successfully achieved by PFGE for the *L. acidophilus* complex, *L. casei*, *L. delbrueckii*, and its three subspecies (*bulgaricus*, *delbrueckii* and *lactis*), *L. fermentum*, *L. helveticus*, *L. plantarum*, *L. rhamnosus* and *L. sakei* (Roussel et al., 1993; McCartney et al., 1996; Klein et al., 1998; Tynkkynen et al., 1999). Drawbacks of method are that it is time-consuming and only a limited number of samples may be analysed.

2.4.3.2. Quantitative molecular methods

Fluorescence *in situ* hybridization

The fluorescence *in situ* hybridization (FISH) technique allows nucleic acid sequences to be examined inside a cell without altering the cell's morphology or the integrity of its various compartments (Amann et al., 1995). The most commonly used target molecule for FISH is 16S rRNA. In the FISH technique, the detection of rRNA sequences within morphologically intact cells is achieved using fluorescently labeled oligonucletide probes.

The procedure includes the following steps: the fixation of the specimen (e.g. using paraformaldehyde), to maintain the integrity of the cell; the permeabilisation, which allows the probe to enter the cells, preparation of the samples, possibly including specific pretreatment steps; the hybridization with the respective probes for detecting the respective target sequence; washing steps to remove unbound probes; mounting, visualisation, counting and documentation of results (Amann et al., 2001). For some gram-positive bacteria, especially lactobacilli, additional pretreatment including the use of cell wall lytic enzymes, e.g. lysozyme, mutanolysin, lyzostaphine, proteinase K or a mixture of these is needed (Harmsen et al., 1999). The multiple rRNA molecules present in bacterial cells capture the probe, the signal is intensified and the cells can be detected and enumerated by using fluorescence microscope or flow cytometry (Jansen et al., 1999; Ben Amor, 2004)

The highly conserved regions of 16S rRNA may be used to design domain-specific probes such as EUB338 which collectively targets most of the bacteria, whereas specific probes for each taxonomic level, down to genus-specific and strain specific probes can be designed according to the highly variable regions of the 16S rRNA (Amann et al., 1990, 1995, 2001). The number of cells containing enough rRNA to be detected by the probe and the total number of cells stained with DAPI (4'6-diamidino-2-phenylindole dihydrochloride) in the same microscopic field are counted.

The first probes were radioactively labeled and hybridization was consequently observed using microautoradiography and it was possible to identify single microbial cells (Giovannoni et al., 1988). Today the nucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked at the 5'-end to a single fluorescent dye molecule. Common fluorophors include fluorescein, tetramethylrhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5 (Southwick et al., 1990).

During recent years, hybridization with rRNA-targeted probes has provided a significant knowledge about the structure of gut microbiota. FISH of the 16S rRNA has shown that species composition is comprised of less than 20 dominant phylogenetic groups (Amann et al., 1990; Alm et al., 1996). Specific nucleotide probes targeting rDNA have been designed for the different species of *Bifidobacterium* and *Lactobacillus* that occur in human intestine. FISH is applied to morphologically intact cells and thus provides a quantitative measure of target organism without the limitation of culture-dependent methods (Amann

et al., 1995, 2001). Presently, probes are available for all *Bifidobacterium* species present in the human intestine. However, the probe panel for lactobacilli is incomplete and lacks specific probes for intestinal species. While lactobacilli are phylogenetically heterogenous, two group-specific probes (Lab 158 and Lab 722) have been designed that cover also related genera such as *Enterococcus, Streptococcus, Vagococcus* and *Oenococcus* (Harmsen et al., 1999).

The major advantage of this method is the capability to detect individual cells in a complex mixture of cells without the need to grow them. However, accessibility is the main problem of this method resulting in low signal intensity. The rRNA may not be fully denaturated, but proteins still interact with it and consequently, many regions may be weakly accessible or even inaccessible to the probe (Fuchs et al., 1998). This problem can be solved by unlabled helper probes which increase the accessibility for the fluorescent probe (Fuchs et al., 2000). There are studies where the composition of the intestinal *Lactobacillu*s spp. has been compared using bacteriological, biochemical and molecular, particularly FISH methods (Harmsen et al., 1999).

Real-time PCR

Another way for direct quantification of the number of bacteria in a sample is to use quantitative real-time PCR (RT-PCR). This method uses a chemiluminscent fluorescent reaction to determine the kinetics of product accumulation during PCR amplification with specific primers for a specific group or species of bacteria. It is then possible to use the product accumulation rate curves to back calculate to the number of original target molecules in a sample. There are different approaches for RT-PCR. One involves using DNA binding dyes such as SYBR Green I which are easy to design and optimise in an assay. Compared to non specific chemistries for RT-PCR such as SYBR Green I, a higher level of detection specificity is provided by using an internal probe together with a primer set to detect the product of interest. In the absence of a specific target sequence in the reaction, the fluorescence probe does not hybridize and therefore the fluorescence remains quenched. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle (Wittwer et al., 2001).

This method is limited by the specificity of the primers used during PCR and has mainly been used to detect and quantify bacteria at the genus or phylum level. The 16S rRNA gene is commonly used as a target molecule however, due to heterogenous gene copy numbers in some species, quantification based on 16S rRNA gene copies can be misleading.

The advantages of this technique are high throughput, high specificity and sensitivity and the absence of requirement of any post-PCR manipulation. The disadvantages are the high sensitivity to contamination, inaccuracies due to differences in DNA extraction or reverse transcription efficiencies, bias introduced by inadequate normalization and the high cost.

Bacterial quantification by RT-PCR can be influenced by differences in the number of rRNA operons between the quantified species or groups, sequence heterogeneity between different operons in the same species, or differential amplification of different DNA molecules (Wintzingerode et al., 1997; Schmalenberger et al., 2001).

The value of real-time PCR has been demonstrated in several studies. Real-time PCR can be used to quantify bacteria from various samples including faeces (Requena et al., 2002; Malinen et al., 2003; Matsuki et al., 2004; Rinttilä et al., 2004, Maruo et al., 2006), dairy products and food (Kao et al., 2007). It can be used for processing, detecting and confirming pathogens in multiple samples at one time (Yanagihara et al., 2010). Recently, RT-PCR has also been applied for strain specific quantification in probiotic products (Vitali et al., 2003; Ahlroos et al., 2009; Kullisaar et al., 2010).

Thus, though several possibilities exist, the develoment of new methods such as microarrays or pyrosequencing hints variability across methods and will require more comparative evaluations.

3. STUDY RATIONALE

In human microbial ecology the species composition of LAB in lower parts of GI tract is still not well described in different age groups or in the case of some environmentally-related diseases such as allergy. This is despite the fact that the main metabolic impact of the microbiota takes place in these regions.

Usually, metabolic impact has been assessed using phenotypic and biochemical analysis. However, molecular analysis provides more precise data. Ideally, extensive phenotypic and biochemical analysis would complement the molecular approach. Nevertheless, a combined approach is thought to be a powerful tool for understanding the true phylogeny and impact of microbes and is emphasised in a review by Palleroni (1997). However, this strategy is too labour intensive for high throughput of organisms of the intestinal ecosystem.

Currently, researchers suggest using the combined analysis of the human genome and its microbiome with the application of functional metagenomics (Gill et al., 2006; Frank and Pace, 2008). Other available approaches are therefore seldom explored. These include the association of variable components of the human microbiota with different clinical and biochemical indices of host (Ouwehand et al., 2009; Tiihonen et al., 2010). It is necessary to provide the level of analysis of the microbiota needed for successful strain selection in order for their application in functional food (de Vos et al., 2006). Although the Food and Agriculture organization (FAO, 2002) has determined a necessity for the data on strain identification, its functional properties and safety, the suggested methods are still voluntarily selected. It is notable that a significant amount of data has been collected on the carbohydrate and amino acid metabolism of lactic acid bacteria and their impact on health-related indices of host.

AIMS OF THE STUDY

The main aims of the study were to characterise the composition and the metabolic impact of intestinal lactic acid bacteria in diverse environments, in people from different age groups, and in the persons with different allergy status using various bacteriological, biochemical and molecular methods.

The present study set the following specific objectives:

1. Comparison of some bacteriological, biochemical and molecular methods for assessment of qualitative and quantitative composition of intestinal lactic acid bacteria and their metabolic properties.
2. Assessment of counts and composition of intestinal *Lactobacillus* spp. in Estonian and Swedish children, healthy Estonian adults and elderly persons.
3. Assessment of association of *Lactobacillus* spp. counts, fermentative groups and species distribution with body mass index and blood glucose content in adults and elderly.
4. Comparison of the intestinal *Bifidobacterium* spp. composition in healthy and allergic 5 years old children by application of genotyping of bifidobacteria.
5. Characterization of genotypic identification pattern and the profile of organic acids, poly- and biogenic amines of a selected probiotic strain *Lactobacillus plantarum* Inducia DSM 21379.

4. MATERIALS AND METHODS

4.1. Subjects and materials

Altogether 71 Estonian infants, 73 Swedish infants, 40 children at the age of 5 (20 allergic, 20 non-allergic), 24 healthy adults and 37 elderly persons were recruited in this study.

The number of *Lactobacillus* sp. strains investigated was 133 (including 35 randomly collected intestinal strains, 76 intestinal strains from 1–2 year old Estonian and Swedish children, 21 reference strains and 1 strain of *L. plantarum* Inducia).

The strain *Lactobacillus plantarum* Inducia DSM 21379, was isolated from a faecal sample of a healthy Estonian child during a comparative study of the microbiota of Estonian and Swedish children.

A summary of the materials and methods used in the study is presented in Table 3.

4.2. Sample collection

The selection of individuals included in our studies and their demographics have been described by coauthors in the aforementioned studies.

Paper I, III: **Identification and characterisation of *Lactobacillus* isolates from the faecal samples of 1–2 year old Estonian and Swedish children.** The collection of specimens was carried out in Tartu, Estonia, from October to December 1995 and in Linköping, Sweden, from March to December 1995 (Sepp et al., 1997).

Paper II, IV: **The prevalence and counts of lactic acid bacteria; bacteriological and molecular methods used for samples from infants and adults**: Altogether 64 faecal samples from the infants and adults. Faecal samples from the infants (n=40) were collected at the Linköping University hospital between December 1996 and August 1998. Faecal samples were collected at 5–6 days and at one, three, six and twelve months of age. The selection criteria included vaginal delivery at term, an uncomplicated perinatal period and the normal birth-weight (range 2965–4320 g, median 3380 g) (Sepp et al., 1997). Faecal samples (n=24) from healthy adults have been included from the study assessing the impact of a probiotic product (ISRCTN38739209). The inclusion criteria for adults were: considering themselves healthy, no gastrointestinal disorders, and no recent history of antibiotic treatment.

Table 3. Study subjects, microbial strains and performed investigations.

Bacteria	Study subjects/No. of persons	Strains/Samples	Methods	Aims	Papers
Lactobacillus species from the culture collection of Dept. of Microbiology		35 intestinal LB isolates	Identification using – API 50CHL – ITS-PCR – GLC (organic acids in microaerobic and anaerobic milieu)	Comparison using different methods for identification of LB	Paper I
		21 LB reference strains			Present study
	71 EST (40F; 31M) and 65 SWE (31F; 34M) 1–2-year-old children	76 intestinal LB isolates		Estimation of geographic differences in LB colonisation	Paper III
L. plantarum Inducia DSM 21379 from the culture collection of Dept. of Microbiology, licensed to Bio-Competence Centre of Healthy Dairy Product LLC	Estonian healthy child	Faecal sample, 1 strain	Identification using – API 50CHL – ITS-PCR – PFGE – Sequencing (16S rRNA) – GLC (organic acids, polyamines)	Genotypic and metabolic properties of the probiotic strain *L. plantarum* Inducia	Paper VI

Table 3. Continued

Bacteria	Study subjects/No. of persons	Strains/Samples	Methods	Aims	Papers
	Probiotic cheese	Cheese sample		Detection of polyamines in probiotic cheese	
Lactobacillus spp., *Bifidobacterium* spp., *Enterococcus* spp.	8 infants 1-year-old (6M; 2F)	40 faecal samples (1w, 4w, 12w, 24 w, 48w) 6 LB isolates	– Cultivation of LAB – FISH – ITS-PCR	Comparison using different methods, the prevalence and the counts of intestinal LAB	Paper II
Lactobacillus spp.	24 healthy adults (15F; 9M)	24 faecal and blood samples	Enumeration of LB – cultivation – FISH Identification using – RT-PCR – LB species-specific PCR – GC (polyamines)	Prevalence of LB species in human gut and their association with BMI and blood glucose	Paper IV
		24 urine samples		Detection of polyamines in urine samples in probiotic trial	Paper VI
	37 healthy elderly (21F; 16M)	37 faecal and blood samples			
Bifidobacterium spp.	40 5-year-old children (23F; 17M; 20 allergic and 20 non-allergic)	40 faecal samples	Identification using – PCR-DGGE – Cloning and sequencing of 16S rRNA gene amplicons	Comparison of intestinal microbiota and the prevelence of bifidobacteria in allergic and non-allergic children	Paper V

EST – Estonian; SWE – Swedish; LB – lactobacilli; F – female; M – male; w – week; BMI– body mass index

Paper IV: **Diversity and metabolic impact of intestinal *Lactobacillus* spp. in healthy adults and elderly.** Faecal and blood samples from 61 persons (24 adults and 37 elderly persons) were collected for this study. The baseline values of the healthy adults have been included from the study assessing the impact of a probiotic product (ISRCTN38739209). The inclusion criteria for adults were: considering themselves healthy, no gastrointestinal disorders, and no recent history of antibiotic treatment.

The healthy elderly persons were selected from the registry of family doctors and orthopedists of Tartu University Hospital, Estonia, before performing the elective orthopedic surgery. The inclusion criteria for the volunteers were: the age 65 years or above, considered themselves generally healthy, no antibiotic treatment.

The blood samples were obtained in the early morning after 8h fasting. The samples were drawn from the antecubital vein with vacutainer into heparinised tubes and immediately stored at 4°C. Plasma glucose (mmol/l) was determined using standard laboratory methods a certified assay in the local clinical laboratory of the Tartu University hospital. Body mass index (BMI) was calculated as the weight (kg) divided by height squared (m^2) (Wenzel, 2007).

Paper V: **Investigation of the predominant bifidobacterial species of the intestinal microbiota in allergic and non-allergic 5–year-old children.** The study group comprised of 40 Estonian children who were at the age of 5 years randomly selected from a larger group in which the immune responses to allergens and the development of allergy were studied starting from birth (Julge et al., 2001). The inclusion criteria were vaginal delivery, breastfeeding at least till the end of neonatal period, presence or absence of the diagnosis of allergy at the age of 5 years and availability of faecal samples. The diagnosis of allergy was based on the clinical examination of the children and on the data obtained from questionnaires (Julge et al., 2001). Nonallergic children had no signs of clinical allergy at any time during the first 5 years of life.

Paper VI: **Detection of bio- and polyamines in urine samples of adults and in a cheese sample**. Altogether 24 samples of morning urine (mid flow) of adults were collected to plastic cups by the staff of Bio-Competence Centre of Healthy Dairy Product LLC. The samples were stored frozen at –20°C until analysis.

Approximately 1–2 g of fresh human faecal sample was collected into the sterile plastic containers. The samples collected at home were kept in a domestic refrigerator at 4°C for no more than 2h before transportation to the laboratory, where the plastic cups were stored frozen at –20°C or –70°C (which depended on time of storage) until use. The Swedish samples were frozen at –20°C immediately at home, delivered to the laboratory within 1 months and then transported on dry ice to Estonia for analysis.

4.3. The reference strains

Thirty one culture collection strains were purchased from both the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikro-organismen und Zellkulturen (DSMZ) and used in the study (Table 4**).**

Table 4. The reference strains used in the study.

Species	**Strain**
L. acidophilus	ATCC 4356
L. delbrueckii ssp. *delbrueckii*	ATCC 9649
L. delbrueckii ssp. *lactis*	DSM20355
L. delbrueckii ssp. *bulgaricus*	DSM20080
L. salivarius	DSM *20555*
L. crispatus	DSM 20584
L. johnsonii	DSM 10533
L. gasseri	DSM 20077
L. curvatus	ATCC 2560
L. helveticus	DSM 20075
L. paracasei	DSM 20020
L. paracasei	DSM 5622
L. paracasei	DSM 13434
L. casei	NCDC 150
L. rhamnosus	ATCC 53103
L. plantarum	DSM 9843
L. plantarum	DSM21380 Tensia
L. plantarum	ATCC 14917
L. sakei	DSM 20100
L. fermentum	ATCC 14931
L. reuteri	DSM 20016
L. cellobiosus	DSM 20055
L. brevis	DSM 20054
L. buchneri	DSM 5987
L. ruminis	DSM 20403
B. longum	DSM 14583
B. lactis	Bb 12 Chr.Hansen, Hørsholm, Denmark
E. coli	ATCC 700336
E. coli	ATCC 700414
E. coli	ATCC 25922
P. aeruginosa	ATCC 27853

4.4. Bacteriological methods for identification of lactic acid bacteria

Weighed faecal samples were serially diluted (10^{-2}–10^{-9}) in pre-reduced phosphate buffer (pH=7.2) in an anaerobic glove chamber (Sheldon Manufacturing, Inc., Shel LAB, USA) with a gas mixture of $CO_2/H_2/N_2$:5/5/90% and cultivated on freshly prepared media: de Man-Rogosa-Sharpe agar (MRS; Oxoid) for microaerophiles, such as lactobacilli; Columbia agar with colistin and nalidixic acids (CAN; BBL) for gram-positive anaerobes, such as bifidobacteria; Yeast extract agar was used for aerobes, particularly enterococci. Incubation of CAN was performed for 5–6 days in an anaerobic glove box. The MRS medium was incubated under microaerobic conditions in a CO_2-thermostat ("Jouan" IG 150, France) for 72 hours; the yeast agar was incubated at 37°C for 48 hours. The colonies of different morphologies growing on the plate with the highest dilution of bacteria were counted, Gram-stained and subjected to the light microscopy.

The intestinal *Lactobacillus* isolates were identified based on a negative catalase reaction, in addition to their colony and cell morphology. The ability of isolates to grow in MRS broth for 24 h in a 10% CO_2 environment at 15°C and at 37°C and to produce gas in MRS agar with 1% glucose was also assessed. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies OHOL; growth both at 15 and 37°C without gas production is characteristic of FHEL, whereas gas production at 37°C and variable growth at 15°C are characteristic of OHEL *Lactobacillus* species (Kandler et al., 1986; Lenzner et al., 1984; Sharpe et al., 1981). The isolated lactobacilli strains were further characterised by API 50CHL kit and analysed by API LAB Plus software version 4.0 database (bioMérieux, Marcy l'Etoile, France).

Bifidobacteria were identified to the genus level based on their growth on selective media (blood agar medium) in anaerobic conditions, colony and cell morphology and Gram-strain reactions.

Enterococci were identified based on the negative catalase rection and differentiated to the genus level by fermentation of aesculine (Facklam et al., 1991).

Subsequent to the identification of microorganisms (qualitative composition), the quantitative composition, counts of microorganisms were determined ($\log_{10}$ CFU/g-colony forming units per gram). A detection level of various microorganisms was 3 log CFU/g.

4.5. Metabolitic activity of lactic acid bacteria

4.5.1. Extraction and detection of organic acids

The production of organic acids and ethanol (mmol/L) was estimated using GC as described by Holdeman et al. (1977). Analysis was performed after cultivation of lactobacilli at 37°C in modified MRS broth for 24 and 48 hours in a 10% CO_2 environment and also in an anaerobic glove chamber (Sheldon Manufacturing, Inc., Shel LAB, USA) with a gas mixture of $CO_2/H_2/N_2$:5/5/90%. The samples were then centrifuged at1500 rpm for 10 min.

For analysis of volatile organic acids an ether extract was prepared by adding 0.2 ml 50% sulfuric acid, 0.4 g sodium chloride and 1.0 ml *tert*- butyl-methyl ether to 1 ml culture broth. The sample was cenrtifugated at 1500 rpm for 3 min. The top layer was removed and drove by adding of calcium chloride. One microliter of the extracted ether phase was injected into the column.

Non volatile organic acids methylate samples were prepared by adding 0.4 g sodium chloride, 0.2 ml 50% sulfuric acid and 2 ml methanol in 1 ml prepared culture broth. The tubes were heated at 60 °C for 1.5h, after which 1 ml of water and 0.5 ml of chloroform were added. The emulsion formed was centrifuged at 3000 rpm for 3 min. One microliter of the chloroform sample was injected into the GC column.

The HP Chemical Station for GC system (A.06 revision) was used. The gas chromatograph (Hewlett-Packard 6890, USA) was equipped with a hydrogen flame ionization detector, an autosampler (model 7683) and a capillary GC column HP-INNOWax (Hewlett-Packard, USA) was 15 m X 0.25 mm (i.d.), coated with cross-linked polyethylene glycol (film thickness 0.15 mm). The detector temperature was 350°C and the injector temperature was 200°C, respectively. The oven temperature program was a gradient system; the initial temperature was 60°C for 1 min that was increased to 120°C at a rate of 20°C/min and held there for 10 min. Acetic, lactic, succinic acids and ethanol (Aldrich, USA) were used as a standards. The stock solutions were a different concentration of each acid and ethanol, namely 20, 50, 100, 1000 mg/L. Each standard solution was injected in duplicate to obtain its retention time and area under the curve. The chromatographic peak areas were integrated with a Hewlett-Packard networking integrator.

4.5.2 Detection of poly-and biogenic amines

GC analysis was performed with a Hewlett-Packard HP model 6890 gas chromatograph (Hewlett Packard, USA) equipped with a split/splitless capillary inlet system and a flame ionization detector (FID). The GC column was a 30 m X 0.32 mm (i.d.) fused silica capillary coated with cross-linked 5% phenyl methyl silicone (film thickness 0.25 mm). The detector temperature was 350°C and the injector temperature was 200°C, respectively. The oven temperature

program was a gradient system; the initial temperature was 150°C that was increased to 280°C at a rate of 20°C/min and held there for 4.5 min. The chromatographic peak areas were integrated with a Hewlett-Packard networking integrator.

A growth medium and cheese: Microbial strains were suspended in physiological saline according to McFarlandi turbidity (10^9 CFU/ml) and 0.5 ml of each strain suspension was seeded into decarboxylation medium (á 4.5 ml) with precursor amino acids (0.5%) (arginine, glutamine, lysine, ornitine and histidine) and incubated at 37ºC for 4 days (Bover-Cid et al., 1999).

Ten grams of cheese were weighed, chopped up into very small fragments and placed into plastic bottle, which contained 20 ml of 50% methanol solution (HPLC grade, Aldrich, USA). The solution was homogenized by vortex mixing for 5 minutes. The cheese mix was then incubated at 45ºC for one hour. Thereafter, the extract was cooled to 30 ºC and centrifuged at 4000 rpm for 15 minutes (Nackovich, 2003).

For detection of poly- and biogenic amines 200 µl of medium or supernate of cheese was derivatized for GC analysis using a modified method of Nakovich (Nakovich, 2003).

Carbamate derivatives of the biogenic amines listed above were created using propyl chloroformate (HPLC grade, Aldrich, USA) as the derivatizing reagent. To a 200 µl of medium (or cheese supernate), 200 µl of a 1:4 mixture of chloroform (HPLC grade; Reanal, Hungary) and iso-octane (HPLC grade, Aldrich, USA) was added to the plastic sample preparative vial. To ensure that the solution remained at a constant pH, 50 µl of pH 12.2 K_2CO_3-$KHCO_3$ buffer was also added to the mix. Finally, 1 µl of propyl chloroformate (98% purity, Aldrich, USA) was added to the solution. The solution was homogenized using a vortex mixer (Heidolph Reax 2000, Germany) for 1 minute. Thereafter, a sample vial was placed in the centrifuge (Eppendorf 5415D, Fisher Scientific Inc., USA) for 5 minutes. Prior to GC analysis, 100µL of the organic layer on top was diluted by removing and depositing it into a glass sample vial, containing 400µL of the chloroform/iso-octane solvent mixture. One microliter of this solution was injected into the GC. Polyamine concentrations in medium and cheese were expressed in mg/L.

Urine: Urine samples (1 ml) were mixed using vortex with 1ml of dichloromethane containing 5µL propylchloroformate. After the first mixing for 10 minutes, pH of the mixture was increased to above 12 with 5M sodium hydroxide solution and the sample was mixed for next 10 minutes. The aqueous phase was removed and the organic phase was centrifuged at 3000 rpm for 5 minutes and the remaining aqueous phase was removed. Subsequently, the organic phase was concentrated under stream of nitrogen, and 1 µL was injected to the GC system. The calibration graphs were prepared using polyamine standard solutions at different concentrations (3–150 nmol). 1,6-diaminohexane was used as internal standard. Polyamine concentrations in urine were expressed as

ng/mol of creatinine. Creatinine was measured colorimetrically using Jaffe kinetic method (Burtis et al., 1998).

4.6. The molecular methods for the detection of lactic acid bacteria

4.6.1. DNA extraction

The DNA extraction from *Lactobacillus* isolates was performed as described by Alander et al. (1999). Bacterial cells were collected from 1 ml of an overnight culture by centrifugation, washed with 50 mM Tris buffer (pH 8.0), and suspended in 100 µl of 50 mM Tris-EDTA buffer (pH 8.0). Lysozyme (100 µl, 20 mg/ml) (Serva, Sweden) and mutanolysin (8 µl, 0.5 mg/ml) (Sigma, USA.) were added, and the mixture was incubated at 37°C for 1 h. The cells were lysed by addition 20 µl of 20% sodium dodecyl sulfate and 12 µl of proteinase K solution (14.6 mg/ml) (Fermentas, Lithuania) followed by a 10 min incubation at 65°C. The volume was adjusted to 500 µl by ultrapure water. Deproteinization was done by extraction with 1 volume of Tris-saturated phenol (Sigma, USA). The water phase was extracted once more with phenol-chloroform (1:1). Finally, DNA was precipitated by adding 0.1 volume of 3M sodium acetate to the water phase followed by 2 volumes of 94% ethanol and incubating the mixture in an ice bath for 30 min. DNA was collected by centrifugation at 13000 rpm for 15 min, and the pellet was washed with 70% ethanol and finally dissolved in 20 µl of sterile ultrapure water.

Bacterial DNA from faecal samples was extracted using at QIAamp DNA stool mini kit (QIAgen, Hilden, Germany) with some modifications. 0.22g of faeces was placed into the ion different bead-beating tubes filled with 0.3g of 0.1 mm zirconia/silica beads and then 1.4 ml of ASL solution from the stool mini kit was added. The tubes were then agitated for 3 min at a speed of 5000 rpm in a mini bead beater (GlenMills Inc., USA). The protocol was then continued from step 3 as described by the manufacturer. The amount of DNA was determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide (Zoetendal et al., 2001).

The amounts of DNA were quantified using spectrophotometry (Quibit™, Invitrogen, USA).

4.6.2. Primers and probes

Primers and probes used in the study were targeted on the 16S rRNA, 23S rRNA or intergenic spacer region between 16S and 23S rRNA genes (Table 5).

4.6.3. PCR based methods

4.6.3.1. Identification of lactobacilli using ITS-PCR/RFLP analysis

ITS-PCR followed by enzymatic restriction was used to confirm the species identification. The DNA amplification was performed according to Jacobsen et al. (1999) in a reaction volume of 50 µl containing 1xTaq polymerase buffer (Fermentas, Lithuania), 1.5U Taq polymerase (Fermentas), 0.5 µM of each primer (16S–1500F and 23S–32R; DNA Tehnology AS, Denmark (Table 5) (Jacobsen et al. 1999), 200 µM deoxynucleoside triphosphates, 2 µM $MgCl_2$ and 2µl of extracted DNA. Subsequently, The PCR products were restricted as descrbed by Zhong et al., 1998 using a *Taq*I restriction enzyme (10U, 65ºC) (Fermentas).

4.6.3.2. Assessment of lactobacilli in faecal samples

The *Lactobacillus* species-specific qualitative PCR was carried out using primers listed in Table 5 targeted on the 16S and 16S–23S rRNA intergenic spacer region. The primer pair for *L. ruminis* subgroup was specific for *L. ruminis*, *L. animalis*, *L. mali*, *L. salivarius* and *L. satsumensis*. A reaction mixture (50µl) consisted of 10X reaction buffer, a 200 µM concentration of each deoxynucleoside triphosphate, 1µM of each primers, 100 ng of bacterial DNA (extracted from faecal samples) and 1.5 U of HotStar Taq Plus DNA polymerase (QIAgen, Germany). The amplification program consisted in pre-denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 30 sec at the appropriate annealing temperature (Table 5), and finally 72°C for 30 sec. A cycle of 72°C for 10 min concluded the program. Amplification products were detected using agarose gel electrophoresis on 2% agarose gel, ethidium bromide staining, and UV transillumination.

The size of the PCR products was compared with that of the aforementioned *Lactobacillus* reference strains (Table 4).

4.6.3.3. Detection of *Bifidobacterium* spp. in faecal samples using PCR-DGGE

4.6.3.3.1. PCR amplification

Bacterial PCR products were produced with the primers 968-GC-f and 1401-r (Lane, 1991). *Bifidobacterium* genus-specific PCR was performed with the primers Bif164-f and Bif662-GC-r (Langendijk et al., 1995; Satokari et al., 2001). Im-3-r and Im-26-f were used for construction of a clone library of 16S rRNA gene from *Bifidobacterium* ssp. (Kaufmann et al., 1997).

PCR was performed in a reaction volume of 50 µl containing 10 mM deoxyribonucleotide triphosphate each, 1.25U of *Taq* polymerase (Invitrogen, USA), 10X reaction buffer, 10 µmol of the each primer (Table 5) and 200 ng (1µl) of DNA solution. The initial DNA denaturation and enzyme activation steps with the primers 968-GC-f and 1401-r were performed at 94°C for 5 min, followed

by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 20 s, and elongation at 68°C for 40 s, which was followed by final elongation at 68°C for 7 min. The annealing temperatures were set at 62°C with the primers Bif164-f and Bif662-GC-r, and at 57°C with the primers Im26-f and Im3-r.

4.6.3.3.2. DGGE analysis of PCR products

The DGGE analysis of PCR amplicons was performed using a Dcode™ System apparatus (Bio-Rad, Hercules, USA). Polyacrylamide gels 8% [wt/vol] acrylamide-bisacrylamide [37.5:1] in 0.5X Tris-acetic acid-EDTA buffers with a denaturing gradient were prepared with a gradient mixer and Econopump (Bio-Rad, USA). Gradients from 30 to 60% were employed for the separation of the products amplified with universal primers and from 45 to 60% for the products amplified with the primers specific for the *Bifidobacterium* spp.

4.6.3.3.3. Cloning of the PCR products

The PCR amplicons were purified and concentrated with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and cloned in *E.coli* JM109 using the pGEM-T vector system (Promega, USA). Colonies for sequencing were selected according to the migration position of the PCR fragment of the clone in DGGE in comparison to the fragments in the original DGGE profile. The plasmid DNA of the selected transformants was isolated using the QIAprep spin miniprep kit (QIAgen, Germany).

4.6.3.3.4. Sequence analysis

Sequencing of the cloned PCR fragments was carried out using purified plasmid DNA and the sequencing primers SP6 and T7 (Promega, USA). The sequencing reactions were performed with a Sequenase sequencing kit (Amersham, Slough, UK) according to manufacturer's instructions. The sequences were analysed with the automatic LI-COR DNA Sequencer 4000L (Lincoln, USA) and corrected manually. Sequence alignment of the complementary strands was carried out using the DNASTAR SeqMan program (Madison, Wis., USA). Similarity searches for the 16S rDNA sequences were performed in the GenBank database using the BLAST algorithm.

4.6.3.4. Real-time PCR

RT-PCR was used to quantify lactobacilli in faecal samples. It was performed with the ABI PRISM 7500 HT Sequence Detection System (Applied Biosystems, USA) using optical grade 96-well plates. The PCR reaction was performed in a total volume of 25 µL using the SYBR Green PCR Master mix (Applied Biosystems, USA). Each reaction included 150 ng of template DNA, 12.5 µL of SYBR Green master mix (Applied Biosystems, USA) and 0.2 µM of

each primer (Table 5). The PCR program consisted of an initial denaturation step 50°C for 2 min and 95°C for 10 min, amplification step followed by 40 cycles consisting of denaturation at 95°C for 15 s, annealing-elongation at 60°C for 1 min and a melting-curve determination step. 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 60–96°C, with continuous fluorescence collection. Negative control was included on each plate.

Standard curves were constructed using a plasmid containing 16S rDNA fragments amplified with the corresponding primers (Rinttilä et al., 2004). The PCR amplicons for *L. paracasei* (DSM 13434) were individually inserted into a separate plasmid vector pGEM-T (Promega Corp., USA); the recombinant vector was transformed into chemically competent *E. coli* JM109 (Promega Corp., USA). The plasmids were purified with the QIAgen MaxiPrep kit (QIAgen, Germany). The purified plasmids were quantified using spectrophotometry (Quibit™, Invitrogen, USA) of multiple dilutions. The copy numbers of the DNA standards were calculated according to Sambrook and Russell et al. (2001). A standard curve for *Lactobacillus* spp. was generated. The target DNA was quantified using serial 10-fold dilutions from 10^3 to 10^9 plasmid copies of the previously quantified plasmid standards. The number of 16S rRNA gene copies that had been previously determined in *Lactobacillus* species (Dudez et al., 2002; Furet et al., 2004) was used to convert the copy numbers into a cell equivalent value (Margin et al., 2006). Plasmid standards and samples were run in triplicate and the average values were used to calculate the bacterial load. The conversion of Ct (cycle threshold) of DNA copy number was based on a linear regression equation of DNA standard curve obtained from each RT-PCR assay.

To check the reliability of the QIAamp DNA Stool Mini kit (Qiagen) and to determinate a detection limit of the RT-PCR method, a recovery experiment was performed. Faecal samples, in which *Lactobacilli* could not be detected, were dosed with aliquots of different dilutions of lactobacilli (ATCC 43597; 10^6–0 CFU/ml). DNA isolation was undertaken and RT-PCR analysis of the samples was performed to determine the amount of lactobacilli. Additionally, electrophoresis on agarose gel was performed to confirm the results.

Data analysis was conducted with the Sequence Detection Software version 1.6.3 (Applied Biosystems, USA). The functions describing the relationship between Ct (threshold number) and x (copy number) for lactobacilli were: $Ct=-3.14x+49.88$; $R^2=0.99$.

4.6.4. Fluorescence *in situ* hybridization

The faecal samples were weighed and phosphate-buffered saline (PBS) added to make up a 1/10 solution (w/V). The sample was homogenized and a 5 ml portion removed and vortexed with glass beads for 30 s, then centrifuged at 300Xg

for 1 min. Bacterial cells were fixed in a 4% (v/v) paraformaldehyde solution overnight at 4 °C and FISH performed as described by Harmsen and associates (Harmsen et al., 1999, 2000). The cells were then washed twice in PBS and resuspended in 1 ml of PBS: ethanol (1:1, v/v). A portion of the cell suspension was hybridized overnight in hybridization buffer (20 mM Tris-HCl, 0.9% NaCl) with Cy3-labeled probe **(**Table 5**)**. The cells were washed with hybridization buffer, filtered through a 0.2 μm polycarbonate (Millipore Corporation) and mounted on a glass slide. Digital images of the slides, viewed with a «Zeiss Axio 2» (Germany) fluorescence microscope, were taken with «Sony 3 CCD» (Japan) device camera. Ten to twenty fields with a total of approximately 200–300 positive cells were counted for each sample.

4.6.5. Pulse field gel electrophoresis

The preparation of genomic DNA *in situ* in agarose was performed using slightly modified version of the method desribed by Simpson (2003). *Lactobacillus* strains were grown to an A_{620} of 0.530 in MRS broth. The cells were harvested from 1.5 ml of culture, washed with 75mM NaCl, 25mM EDTA (pH 7.4), and suspended in 150 μl of the same buffer. The suspension was heated to 50°C, and 150 μl of 2% agarose in 0.5XTBE buffer at the same temperature was added before solidifying the suspension in molds. The agarose plugs were incubated overnight at 37 °C in lysis buffer (50mM EDTA (pH 8.5), 0.5% Na-laurylsarcosine, 0.2% Na-deoxylate) containing 2mg of lysozyme (Sigma) per ml and 10U of mutanolysin (Sigma) per ml and then incubated overnight at 53 °C in solution containing 10 mM Tris, 0.5M EDTA (pH 8.5), 1% sodium dodecyl sulfate and 2 mg of proteinase K per ml. The agarose blocks were washed three times with 75mM NaCl, 25mM EDTA (pH 7.4). A restriction enzyme digestion with *NotI* was performed overnight at 37 °C. Electrophoresis was carried out with a CHEF DR II (Bio-Rad, Hercules, USA) with 0.5XTBE buffer. The pulse time was 1 to 15 s, the current was 6V/cm, the temperature was 14°C, and the running time was 18 hours. The agarose gel was stained with etidium bromide (0.5μg/ml) and visualised under UV light.

4.7. Statistical analysis

The statistical analysis was performed using SIGMASTAT 2.0 (Jandel Scientific Corporation, USA) and SPSS 11.0 (SPSS Inc., Chicago, IL; USA) statistical software packages. Data were presented as the mean value ± SD or range and median depending on the normality distribution of data. Bacterial counts data were logarithmically transformed; the prevalence of the species was described as percentage (%).

Table 5. List of probes and primers used in the study.

Primers	Sequence (5'– 3')	Specificity of target	Methods	Annealing temp (°C)	Reference
Lab 158	GGTATTAGCA[C/T]CTGTTTCCA	*Lactobacillus* 16S	FISH	45	Harmsen et al., 1999
Bif 164	CATCCGGCATTACCACCC	*Bifidobacterium* 16S	FISH	50	Langendijk et al., 1995
968 GC-F	CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGG-AACGCGAGAACCTTAC	*Eubacterial* 16S	PCR	56	Nubel et al., 1996
1401-R	GCGAAGAACCTTAC	*Eubacterial* 16S	PCR	56	Nubel et al., 1996
CO1-F	AGTTTGATCCTGGCTCAG	*Eubacterial* 16S	Sequencing	52	Simpson et al., 2003
CO2-R	TACCTTGTTACGACT	*Eubacterial* 16S	Sequencing	52	Simpson et al., 2003
T7	GTGAAGCTTACGGT[C/T]TACCTTGTTAC GACTT	pGEM-^{T}	Sequencing	44	Promega
SP6	TAATACGACTCACTATAGG	pGEM-^{T}	Sequencing	44	Promega
Bif-164-F	GATTTAGGTGACACTATAG	*Bifidobacterium* 16S	PCR/ nested	62	Kok et al., 1996
Bif-662-GC-R	CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGGG-CCACCGTTACACCGGGAA	*Bifidobacterium* 16S	PCR/ nested	62	Kok et al., 1996
Im-3-R	GGGGGG-CCACCGTTACACCGGGAA	*Bifidobacterium* 16S	PCR/ nested	57	Kaufmann et a., 1997
Im-26-F	CGGGTGCTICCCACTTTCATG	*Bifidobacterium* 16S	PCR/ nested	57	Kaufmann et a., 1997
1500-F	GATTCTGGCTCAGGATGAACG	*Lactobacillus* (16S-23S)	ITS-PCR	48	Jacobsen et al. 1999
32-R	AGGTCCTAACAAGGTA	*Lactobacillus* (16S-23S)	ITS-PCR	48	Jacobsen et al. 1999
F_acid_IS	GAAAGAGCCCAAACCAAGTGATT	*L. acidophilus* 16S-23S	PCR	59	Haarman et al., 2006

Table 5. Continued

Primers	Sequence (5'– 3')	Specificity of target	Methods	Annealing temp (°C)	Reference
R_acid_IS	CTTCCCAGATAATTCAACTATCGCTTA	*L. acidophilus* 16S–23S	PCR	59	Haarman et al., 2006
F_helv_IS	CTCTTCTCGGTCGCCTTG	*L. helveticus* 16–23S	PCR	56	Tilsala-Timisjärvi et al. 1997
R_helv_IS	GAAGTGATGGAGAGTAGAGATTA	*L. helveticus* 16–23S	PCR	56	Tilsala-Timisjärvi et al. 1997
R_delb_IS	CGAACTCTCTCGGTCTT	*L. delbrueckii* 16–23S	PCR	58	Haarman et al., 2006
F_delb_IS	CACTTGTACGTTGAAAACTGAATATCTTAA	*L. delbrueckii* 16–23S	PCR	58	Haarman et al., 2006
R_sal_IS	GTCGTAACAAGGTAGCCGTAGGA	*L. salivarius* 16S	PCR	61	Harrow et al., 2007
F_sal_IS	TAAACAAAGTATTCGATAAATGTACAGGTT	*L. salivarius* 16S	PCR	61	Harrow et al., 2007
R_cri_IS	GTAATGACGTTAGGAAAGGG	*L. crispatus* 16–23S	PCR	57	Walter et al., 2000
F_cri_IS	ACTACCAGGGTATCTAATGG	*L. crispatus* 16–23S	PCR	57	Walter et al., 2000
R_joh_IS	GAGCTTGCCTAGTAGATTTTA	*L. johnsonii* 16–23S	PCR	58	Song et al., 2000
F_joh_IS	ACTACCAGGGTATCTAATCC	*L. johnsonii* 16–23S	PCR	58	Song et al., 2000
R_gas_IS	AGCGACCGAGAAGAGAGAGA	*L. gasseri* 16–23S	PCR	57	Song et al., 2000
F_gas_IS	TGCTATCGCTTCAAGTGCTT	*L. gasseri* 16–23S	PCR	57	Song et al., 2000
F_rum_sub	CACCGAATGCTTGCAYTCACC	*L. ruminis* subgroup	PCR	57	Matsuda et al., 2009
R_rum_sub	GCCGCGGGTCCATCCAAAA	*L. ruminis* subgroup	PCR	57	Matsuda et al., 2009
R_ferm_IS	ACTTAACCTTACTGATCGTAGATCAGTCA	*L. fermentum* 16–23S	PCR	58	Haarman et al., 2006
F_ferm_IS	AACCGAGAACACCGCGTTAT	*L. fermentum* 16–23S	PCR	58	Haarman et al., 2006
F_reut_IS	ACCGAGAACACCGCGTTATTT	*L. reuteri* 16–23S	PCR	59	Haarman et al., 2006

Table 5. Continued

Primers	Sequence (5'– 3')	Specificity of target	Methods	Annealing temp (°C)	Reference
R_reut_IS	CATAACTTAACCTAAACAATCAAAGATT GTCT	*L. reuteri* 16–23S	PCR	59	Haarman et al., 2006
F_bre_IS	ATTTTGTTTGAAAGGTGGCTTCGG	*L. brevis* 16–23S	PCR	55	Watanabe, 1998
R_bre_IS	ACCCTTGAACAGTTACTCTCAAAGG	*L. brevis* 16–23S	PCR	55	Watanabe, 1998
R_buc_IS	GGACCAATG GAGCAACTGAA	*L. buchneri* 16S–23S	PCR	55	Stevenson et al., 2005
F_buc_IS	AGATTACTGACGCATTGGTTACCA	*L. buchneri* 16S–23S	PCR	55	Stevenson et al., 2005
R_casei_IS	CTTCCTGCGGGTACTGAGATGT	*L. casei* 16–23S	PCR	59	Haarman et al., 2006
F_casei_IS	CTATAAGTAAGCTTTGATCCGGAGATTT	*L. casei* 16–23S	PCR	59	Haarman et al., 2006
F_paca_IS	ACATCAGTGTATTGCTTGTCAGTGAATAC	*L. paracasei* 16–23S	PCR	60	Haarman et al., 2006
R_paca_IS	CCTGCGGGTACTGAGATGTTTC	*L. paracasei* 16–23S	PCR	60	Haarman et al., 2006
F_plan_IS	TGGATCACCTCCTTTCTAAGGAAT	*L. plantarum* 16–23S	PCR	60	Haarman et al., 2006
R_plan_IS	TGTTCTCGGTTTCATTATGAAAAAATA	*L. plantarum* 16–23S	PCR	60	Haarman et al., 2006
F_rham_IS	CGGCTGGATCACCTCCTTT	*L. rhamnosus* 16–23S	PCR	59	Haarman et al., 2006
R_rham_IS	GCTTGAGGGTAATCCCCTCAA	*L. rhamnosus* 16–23S	PCR	59	Haarman et al., 2006
R_cur_IS	TTGGTACTATTTAATTCTTAG	*L. curvatus* 16–23S	PCR	58	Aymemerich et al., 2003
F_cur_IS	GCTGGATCACCTCCTTTC	*L. curvatus* 16–23S	PCR	58	Aymemerich et al., 2003
R_sak_IS	GAGCTAATCCCCCATAATGAAACTAT	*L. sakei* 16–23S	PCR	57	Martin et al., 2006
F_sak_IS	GATAAGCGTGAGGTCGATGGTT	*L. sakei* 16–23S	PCR	57	Martin et al., 2006
Lac-F	AGCAGTAGGGAATCTTCCA	*Lactobacillus* group 16S	Real-time PCR	58	Walter et al., 2000
Lac-R	CACCGCTACACATGGAG	*Lactobacillus* group 16S	Real-time PCR	58	Walter et al., 2000

Other statistical tests was used, included the odds Ratio (OR) test with 95% confidence intervals (CI 95%), the X^2 or Fisher exact test for the prevalence of LAB, the student's t-test or Mann-Whitney rank sum test for the amount of SCFA and LAB. Logistic regression analysis was performed to compare the binary variables and/or continuous variables. The Spearman rank correlation test and the multiple linear regression models were used to test the associations between the microbiological and clinical or biochemical indices. The models were adjusted for BMI, age, and gender. All differences were considered statistically significant if the $p<0.05$.

5. RESULTS

5.1. Comparison of bacteriological and molecular methods for the identification of intestinal *Lactobacillus* isolates (Papers I, III)

In the study, described in Paper I, the results for identification of the intestinal lactobacilli isolates (n=35) obtained from 1–24 months old children (Sepp et al., 1997, 2000) were compared using two methods: 1) an API 50CHL kit analysed by API LAB Plus software version 4.0 database (bioMèrieux, Marcy l'Etoile, France) and 2) ITS-PCR followed by enzymatic restriction. The concordance of species identification by both methods was 22/35 (71%): OHOL group 4/9; FHEL group 8/11; OHEL group 10/15 (Table 1 from Paper I).

Paper III describes a larger set (n=76) of lactobacilli for comparison of identification results using both the API kit and ITS-PCR. The strains of lactobacilli were obtained from Estonian (mean age 18 months) and Swedish (mean age 16 months) children. The intestinal lactobacilli were divided into 13 species by API 50CHL kit and into 8 species by ITS-PCR (Figure 8).

ITS-PCR confirmed the species identity of different *L. acidophilus* strains from different *L. acidophilus* biotypes assigned by API 50CHL. Furthermore it reassigned 4 of 20 isolates from OHOL group despite good or very good identification levels by API to *L. acidophilus*, *L. gasseri* and *L. paracasei* (Figure 8A).

Within the FHEL group, we found that 5 out of 32 isolates, namely *L. curvatus*, *L. plantarum* and *L. rhamnosus* were reassigned to *L. paracasei* ssp. *paracasei* by API (Figure 8 B).

In the case of OHEL group, 5 out of 24 isolates were reassigned by ITS-PCR to some other species (*L. brevis* 2 to *L. buchneri* (n=3), *L. cellobiosus* to *L. fermentum* (n=1) and *L. buchneri* to *L. paracasei* ssp. *paracasei* (n=1). Three *L. coprophilus* isolates by API were not confirmed by ITS-PCR, as the reference strain was not available for comparison. However, the ITS-PCR profiles of these strains were different from the other OHEL group strains (Figure 8 C).

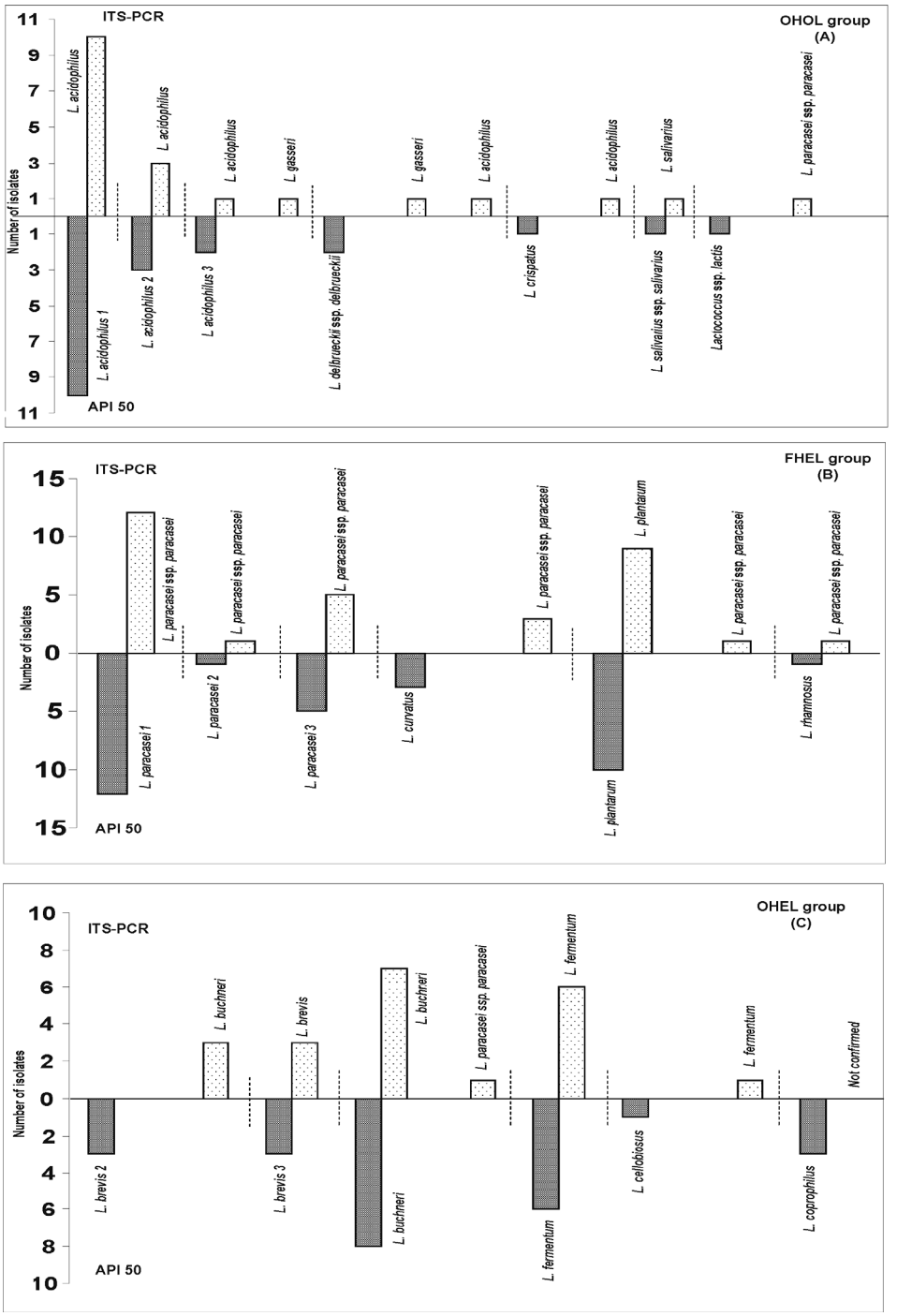


Figure 8. Comparative identification of the intestinal lactobacilli isolates by ITS-PCR (upper part) and API 50CHL (lower part), based on the fermentation groups (OHOL n=20 (A), FHEL n=32 (B), OHEL n=24(C)).

Thus, the concordance with the larger number of faecal isolates of lactobacilli was 62/76 (81.5%), including strains of lactobacilli which remained unidentified by both methods. In the majority of cases, according to ITS-PCR, no difference was found between the biotypes of different species assigned using API 50CHL. However, the gold standard i.e. the identification of the reference strains with both methods was not performed. This was improved in the present study due to the possibility to obtain more reference strains from the international culture collections**.**

Data from the reference strains. The results of API 50CHL and ITS-PCR for reference strains of lactobacilli are depicted in Table 6**.** By API out of 10 OHOL group strains beside *L. salivarius* and *L. delbrueckii* ssp. *lactis* 8 strains were identified as *L. acidophilus* 1 **(**Table 6**).** However, by ITS-PCR only 3 strains were identified as the concordant species. In 6 strains of FHEL group, 4 were assigned *L. paracasei* and both one as *L. curvatus* and *L. plantarum* by API 50CHL, the concordance by ITS-PCR was found in 4 strains. In OHEL group, out of 5 reference strains of *L. fermentum, L. brevis* and *L. buchneri* 4 strains were identified as *L. fermentum* 1, 2 by API, by ITS-PCR the concordance was found only for 3 strains.

Thus, overall concordance between API 50CHL and ITS-PCR was found in 10 out of 21 (48%) reference strains tested by both methods. The reference strains belonged to 19 different species, some of which are quite rare habitants of humans. Overall, the ITS-PCR method showed very good concordance with the reference strains, only *L. delbrueckii* ssp. *lactis* DSM20355 and ssp. *bulgaricus* DSM 20080 were misidentified as *L. delbrueckii* ssp. *delbrueckii*. Moreover, *L. casei* NCDC 150 and *L. cellobiosus* DSM 2005 could not be distinguished from *L. paracasei* and *L. fermentum*, respectively. This suggests the need for a validation of ID of reference strains or finding more appropriate primers for PCR.

Thus, studying a large set of intestinal isolates, the ITS–PCR method was significantly more reliable than API. In the re-evaluation of the data and accepting the reference strains ID as a golden standard the concordance of API 50CHL and ITS-PCR was found only in half of the cases (48%) while the ID of the reference strains and ITS-PCR results concorded in 17/21 (80%). Therefore, in for further work such as an analysis of geographical (Paper III) and age derived differences (Paper IV) we relied on data obtained by ITS-PCR.

Table 6. Comparative identification of lactobacilli reference strains using API 50CHL and ITS-PCR.

Reference strains	Species identification by API	Quality of identification[•]	Identification by ITS-PCR
OHOL group			
L. acidophilus ATCC 4356	*L. acidophilus* 1	Good	***L. acidophilus***
L. salivarius DSM 20555	*L. salivarius*	Excellent	***L. salivarius* ssp. *salivarius***
L. johnsonii DSM 10533	*L. acidophilus* 1	Good	***L. johonsonii***
L. gasseri DSM 20077	*L. acidophilus* 1	Very good	***L. gasseri***
L. jensenii DSM 20557	*L. acidophilus* 1	Doubtful	***L. jensenii***
L. delbrueckii* ssp. *delbrueckii ATCC 9649	*L. acidophilus* 1	Good	***L. delbrueckii* ssp. *delbrueckii***
L. delbrueckii* ssp. *lactis DSM 20355	*L. delbrueckii* ssp. *lactis* 2	Doubtful	***L. delbrueckii* ssp. *delbrueckii*[*]**
L. delbrueckii* ssp. *bulgaricus DSM 20080	*L. acidophilus* 1	Good	***L. delbrueckii* ssp. *delbrueckii*[*]**
L. ruminis DSM 20403	*L. acidophilus* 1	Good	***L. ruminis***
L. helveticus DSM 20075	*L. acidophilus* 1	Very good	***L. helveticus***
FHEL group			
L. curvatus ATCC 2560	*L. curvatus* ssp. *curvatus*	Good	***L. curvatus***
L. rhamnosus ATCC 53103	*L. paracasei* ssp. *paracasei* 1	Unacceptable	***L. rhamnosus***
L. paracasei* ssp. *paracasei DSM 20020	*L. paracasei* ssp. *paracasei* 2	Good	***L. paracasei* ssp. *paracasei***
L. plantarum DSM 9843	*L. plantarum* 1	Very Good	***L. plantarum***
L. casei NCDC 150	*L. paracasei* ssp. *paracasei* 1	Very good	***L. paracasei* ssp. *paracasei***
L. sakei DSM 20100	*L. paracasei ssp.paracasei* 1	Doubtful	***L. sakei***
OHEL group			
L. fermentum ATCC 14931	*L. fermentum* 1	Very good	***L. fermentum*[♦]**
L. buchneri ATCC 4005	*L. fermentum* 2	Excellent	***L. buchneri***
L. brevis ATCC 14869	*L. brevis* 1	Good	***L. brevis***
L. reuteri DSM 20016	*L. fermentum* 1	Good	***L. reuteri***
L. cellobiosus DSM 20055	*L. fermentum* 2	Excellent	***L. fermentum*[♦]**

[•] – Quality of identification derived from the percentage of identification (represents an estimate of how closely the profile corresponds to the stated taxon relative to all the other taxa in the database; T index (represents an estimate of how closely the profile corresponds to the most typical set of reactions for the stated taxon. Identification of selected taxa: **excellent**: %ID 99.9 and T 0.75; **very good**: %ID 99.0 and T 0.5; **good**: %ID 90.0 and T 0.25; **acceptable**: %ID 80.0 and T 0.0. The discrimination is **low** if two or more taxa belonging to the different genera have been selected. The profile is **doubtful** if a taxon having several tests against the identification is present among those selected

[♦,*] – reference strains with similar molecular profiles

5.1.1. Biochemical and molecular identification of probiotic *L. plantarum* strain Inducia DSM 21379 (Paper VI, present study)

Species-specific identification. The selected probiotic strain *L. plantarum* Inducia was identified on the basis of carbohydrate fermentation patterns with API 50CHL System, ITS-PCR, sequencing of 16S rRNA and also PFGE methodology. For assessment of the strain as *L. plantarum*, the API CHL50 System kit, gave the ID% 99.9, *excellent*, with one test contra. The above mentioned strain fermented the following sugars: ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutime, esculine, salicin, cellobiose, maltose, lactose, melibiose, sachharose, trehalose, melezitose, β-gentiobiose, D-turanose and gluconate. A weak reaction for starch was also seen.

The species-specific molecular identification of *L. plantarum* Inducia was performed by ITS-PCR and this data was concordant with the API method. The banding pattern of the isolate was visually compared with that of the reference strains of *L. plantarum* (ATCC 14917, DSM 21380, DSM 9843) (Figure 9 (A)).

Further we identified strain *L. plantarum* Inducia by sequencing of 16S rRNA. Comparison of the 16S rRNA sequences obtained using the BLAST algorithm at the NCBI database (www.ncbi.nlm.nihgov/BLAST/) allowed the assignment of the strain to the particular species. In general, when 16S rRNA similarity values exceed 98%, the strains are considered to belong to the same species. According to sequencing analysis, 16S rRNA of *L. plantarum* Inducia DSM 21379 showed similarity (99%) with *L. plantarum* from the GeneBank database (www.ncbi.nlm.nihgov/BLAST/).

Strain-specific identification. Strain-specific identification of *L. plantarum* Inducia DSM 21379 was performed by PFGE method using the NotI enzyme. Genomic DNA digested with NotI yielded fragments of approximately 48 to 5 kb (Figure 9 (B)). The banding pattern of *L. plantarum* Inducia was different from that of the reference strains of *L. plantarum* ATCC 14917 and some other *L. plantarum* isolates analysed.

The species-specific identification of lactobacilli using various methods, including molecular strain typing, is considered an important parameter for the selection of probiotic strains (Reid et al., 2002; FAO, 2002). We confirmed that the *Lactobacillus* species identification of the bacteriologically obtained isolate was reliable if using a systematic approach with API 50CHL, ITS-PCR and sequencing methods. The strain specific identification distinguishes this probiotic strain from the others and confirms its novelty. It has been deposited in a culture collection (Deutsche Sammlung für Mikroorganismen, DSM) with the acronym Inducia and number DSM 21379. The deposition is important for ensuring the stability of probiotic products, comprised with the selected strain.

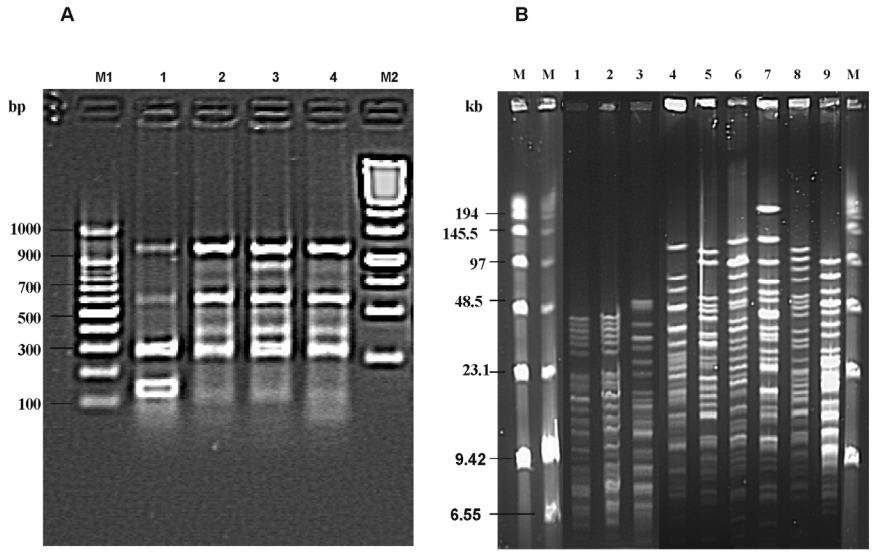


Figure 9. (A) Restriction patterns of *TaqI* digested 16S-23SrRNA intergenic spacer region of *L. plantarum* strains. Lanes: **M1** – 100bp ladder (Fermentas), **1** – *L. plantarum* Inducia DSM 21379, **2** – *L. plantarum* DSM 21380, **3** – *L. plantarum* ATCC 14917, **4** – *L. plantarum* DSM 9843, **M2** – 1kb ladder (Fermentas).
(B) PFGE patterns of *Not*I digested genomic DNA of *Lactobacillus* reference strains and isolates. Lanes: **M** – Low range PFG marker (New England BioLab), **1** – *L. plantarum* Inducia DSM 21379, **2** – *L. plantarum* Inducia isolate from cheese, **3** – *L. plantarum* Tensia DSM 21380, **4** – *L. plantarum* intestinal isolate, **5** – *L. paracasei* DSM 20020, **6** – *L. paracasei* DSM 5622, **7** – *L. plantarum* isolate 317–4, **8** – *L. paracasei* DSM 13434, **9** – *L. plantarum* ATCC 14917.

5.2. Metabolic activity of *Lactobacillus* and *Bifidobacterium* spp.

5.2.1. Production of organic acids and ethanol by lactobacilli and bifidobacteria isolates and its relationship with their culture environment (Papers I, III, and present study)

In total, 21 *Lactobacillus* spp. and 2 *Bifidobacterium* spp. reference strains and 41 faecal isolates of lactobacilli, were analysed for production of organic acids and ethanol by GC in different environments**.** Acetic, lactic, succinic acids and ethanol were the main fermentation products detected. Additionally, as a control, the metabolite profile of culture collection strains used in the study was matched with their fermentation types (Table 7).

The production of organic acids by reference strains did not differ significantly from the lactobacilli isolates tested to the fermentation group level. GC analysis of metabolites produced by lactobacilli in a microaerobic atmosphere

(Table 7) demonstrated that the strains from FHEL group, both reference strains and the intestinal isolates produced abundant lactate, following growth for 48 h.

Differences were found in the production of organic acids from different fermentation groups in the microaerobic and anaerobic environments (Table 7). Higher amounts of acetic and lactic acids were yielded by OHEL and FHEL intestinal strains, respectively. The values for the reference strains corresponded to these of the aforementioned intestinal strains. In addition, significantly higher level of ethanol was produced by OHEL strains grown in a microaerobic vs an anaerobic environment. Besides, within the OHEL group, some strains were able to produce approximately three times more ethanol in an anaerobic milieu compared with the mean of the group. This shows that the particular strains of the same species have different potential for fermentative activity. Moreover, the expression of the genetically determined properties was influenced by the different environmental factors.

***L. plantarum* Inducia**: Our patented strain *L. plantarum* Inducia DSM 21379 (FHEL group) demonstrated a heterofermentative type of metabolism with the fermentation profile exclusively dependent on the environmental conditions (Table 8).

Table 8. Production of organic acids by *L. plantarum* Inducia DSM 21379 in microaerobic and anaerobic environments at 24 and 48 hours (mmol/l).

Environment	Acetic acid		Lactic acid		Succinic acid	
	24h	48h	24h	48h	24h	48h
Microaerobic	2.1	2.4	133.3	186.6	0.6	0.6
Anaerobic	2.9	4.2	93.3	106.6	0.5	0.7

The fermentation profile of *Bifidobacterium* spp. was also dependent on environmental conditions. In an anaerobic environment the main fermentation products of *B. longum* B46 and *B. lactis* B12, after 24h incubation, were acetic (2.5 vs. 37.2 mmol/l) and lactic acids (23 vs. 143.6 mmol/l), respectively. The latter strain clearly deserved its name – *B. lactis* (now reassigned *B. animalis*). No fermentation products were registered in the microaerobic environment due to the absence of growth of the bifidobacteria.

Table 7. Production of organic acids and ethanol by the reference strains and intestinal isolates of lactobacilli after cultivation in microaerobic and anaerobic environments at 24 and 48 hours (mmol/L).

Organic acids/ alcohol	Environments	The amount of organic acids and ethanol (mmol/l) mean± S.D.											
		OHOL				FHEL				OHEL			
		Reference strains (n=10)		Intestinal isolates (n=10)		Reference strains (n=6)		Intestinal isolates (n=13)		Reference strains (n=5)		Intestinal isolates (n=18)	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Acetic acid	Micro-aerobic	1.7±0.6	2.4±1.0	0.8±0.5	1.1±0.4*	1.5±1.2	1.8±0.9	1.0±0.5	1.5±0.4†	3.6±2.3	4.4±2.3	2.4±1.1	5.3±5.0*,†
	Anaerobic	1.6±0.1	1.8±0.05	1.6±0.7	2.1±0.4	2.0±0.1	2.3±0.7	2.2±0.4	2.8±1.0	3.4±1.0	5.1±2.8	3.5±1.4	4.5±1.9
Lactic acid	Micro-aerobic	62.2±27.7	86.5±30.8	50.8±30.6	81.3±27.8‡	75.7±23.1	97.4±32.7	93.2±20.6	129.6±36.4*,§	51.5±33.9	78.9±16.2	52.8±35.7	76.8±23.2$^{\S,\square}$
	Anaerobic	94.7±28.6	102.9±29.7	81.6±21.5	91.1±26.2	85.2±32.9	99.4±38.8	92.6±15.9	113.8±16.1	59.6±27.4	74.4±28.6	33.2±34.8	46.9±36.9$^{\square}$
Succinic acid	Micro-aerobic	0.69±0.15	0.83±0.2	0.5±0.2	0.6±0.2	0.4±0.05	0.5±0.1	0.4±0.1	0.5±0.1	0.45±0.1	0.55±0.1	0.6±0.3	0.8±0.6
	Anaerobic	1.0±0.45	0.83±0.2	1.0±0.6	0.7±0.2	0.4±0.05	0.6±0.05	0.5±0.1	0.7±0.1	0.5±0.1	0.6±0.2	0.5±0.1	0.6±0.2
Ethanol	Micro-aerobic	0	0	–	–	0	0	traces	traces	91.2±59.8	135.3±38.8	98.5±50.1	151.7±26.1**
	Anaerobic	0	0	–	–	0	0	traces	traces	69.5±54.5	75.3±47.2	92.5±125.9	115.8±159.2**

$^{*},^{\dagger},^{\ddagger},^{\S},^{\square}$ a significant difference p=0.001;**a significant difference P=0.003

5.2.2. Production of poly- and biogenic amines by *Lactobacillus* spp. strains (Paper VI, present study)

The production of poly- and biogenic amines (putrescine, cadaverine and histamine) from precursor amino acids (arginine, glutamine, lysine, ornitine and histidine) by 5 lactobacillus and 4 gram-negative strains was tested using GC. *Escherichia coli* strains and *Pseudomonas aeruginosa* were used as controls due to their high levels of production of biogenic amines and putrescine (Table 9).

Lactobacilli, in comparison to enterobacteria, were able to produce putresine (median 0, range 0–2.1 *vs*. median 14.4, range 1.8–2228.5; $p<0.0001$) and cadaverine (median 0.3, range 0–0.6 *vs*. median 11.8, range 0–240; <0.0001) in very small amounts. No production of histamine from histidine by lactobacilli strains was observed.

The production of biogenic amines by 3 strains of *L. plantarum* was strain-specific: they all produced putrescine from ornithine, yet strain Inducia produced it also from glutamine. Thus, the amount of putrescine produced was the highest by the strain Inducia. Traces of cadaverine were produced by the reference strain of *L. plantarum* and by Inducia DSM 21379. No histamine production was detected.

The reference values for food are nearly 1000 mg L $(kg)^{-1}$ that usually cannot be reached by lactobacilli (Karovicoka et al., 2005). Moreover, it was tested in probiotic cheese comprised by Inducia strain where the putrescine and cadaverine values were quite low after 3 weeks ripening (PCT/EE2009/000006; p. 21).

The production of putrescine *in vitro* by *L. plantarum* Inducia was 6 times higher compared to that of *L. plantarum* strain Tensia (3.1 vs. 0.5 mg/L) (Figure 10A). In addition, the production of putrescine in cheese was proportional to that *in vitro* (24.67 vs. 1.32 mg/kg) (Figure 10B). A dose response was seen in human consumers of the cheese who showed the increased values of acetylated putrescine in urine following the probiotic consumption period. Moreover, a significant increase of acPut was found ($p=0.02$) in urine of volunteers consuming cheese with *L. plantarum* Inducia for 3 weeks, which was not registed after consuming a control cheese and cheese with *L. plantarum* Tensia (Figure 10C).

Thus, the amount of putrescine produced *in vitro* by the *L. plantarum* strains (Inducia and Tensia) both from the decarboxylation media and cheese, as assessed by GC, was in a good concordance with its concentration in urine of humans. The strain *L. plantarum* Inducia with the higher concentration in medium and food caused an increase of turn-over of putrescine metabolism in the urine of its consumers.

Table 9. Production of polyamines *in vitro* (μg/ml) by some *Lactobacillus* spp. in decarboxylation media with different precursor amino acids.

Bacteria	Precursor amino acids								
	Arg		Glu		Lys		Orn		His
	Put	Cad	Put	Cad	Put	Cad	Put	Cad	Hist
L. plantarum Inducia (DSM 21379)	0	0.4	1.2	0.5	0	0.4	1.9	0	0
L. plantarum Tensia (DSM 21380)	0	0	0	0	0	0.3	0.5	0.6	0
L. plantarum ATCC 14917	0	0	0	0	0	0.3	0.5	0.6	0
L. plantarum (intestinal isolate)	0	0.3	1.1	0.4	1.6	0.3	2.1	0	0
L. fermentum ME-3 (DSM 14931)	0	0.6	0.8	0.5	0	0.4	1.3	0	0
E. coli ATCC 700336	18.4	1.7	12.3	18.4	1.8	240	1599.3	3.5	105.1
E. coli ATCC 700414	15.3	1.4	13.3	11.3	4.9	11.5	50	0	125.4
E. coli ATCC 25922	12.1	0	23.5	20.4	4.7	10.8	2228.5	3.5	201.8
P. aeruginosa ATCC27853	28.1	1.1	0	0	1.7	22.3	65.3	0	0

Arg – arginine; Glu – glutamine; Lys – lysine; Orn – ornitine; His – histidine, Put – putrescine, Cad – cadaverine, Hist – histamine

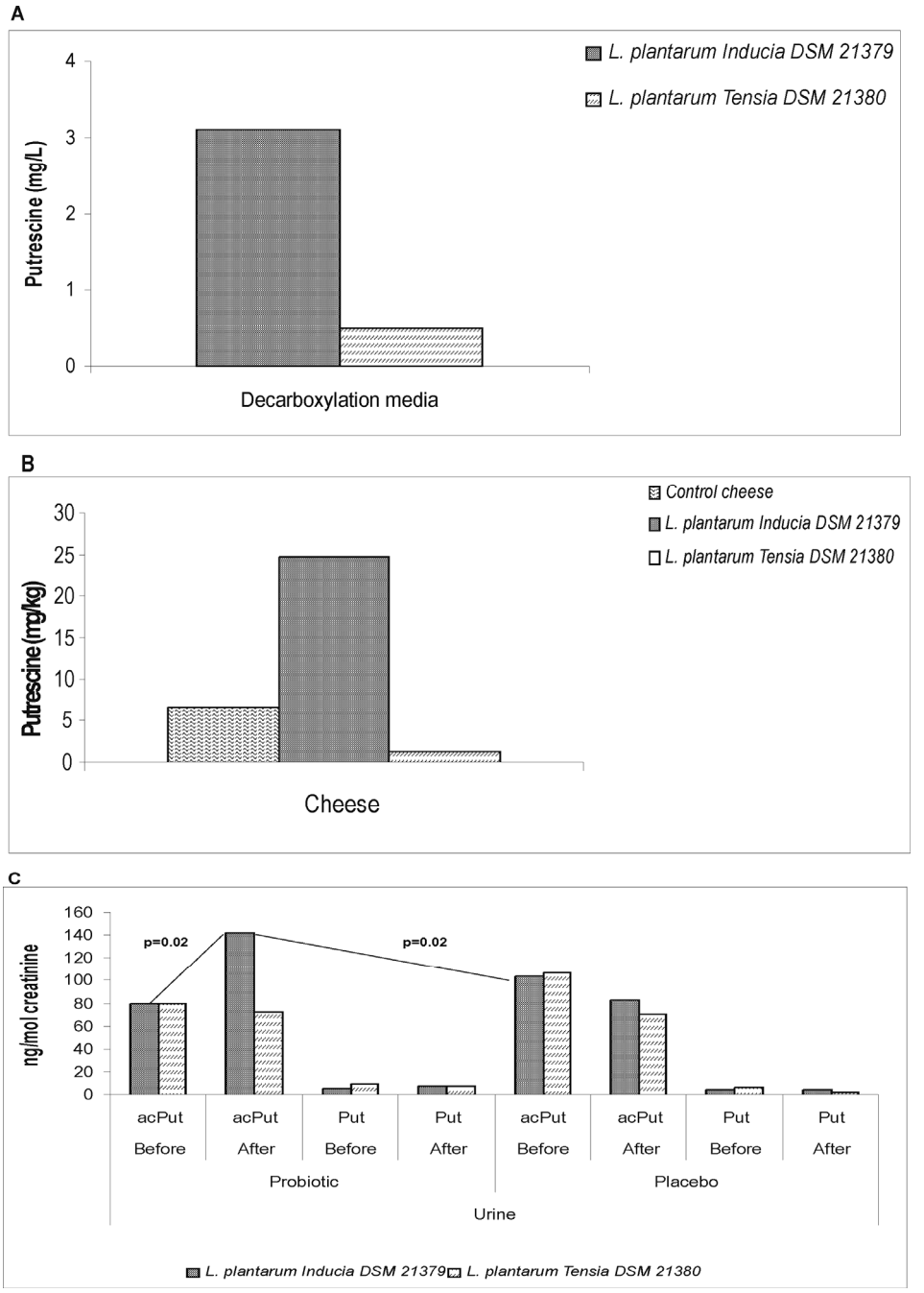


Figure 10. Detection of putrescine (Put) and acetyl-putrescine (AcPut) in decarboxilation media (A), control and probiotic cheeses (B) and in urine (C) of persons consuming probiotic cheeses comprising *L. plantarum* Inducia DSM 21379, *L. plantarum* Tensia DSM 21380 and control cheese.

5.3. The detection and prevalence of lactic acid bacteria determined using bacteriological and molecular methods (Papers II, VI)

In total, 40 faecal samples from infants and 24 from adults were assessed for the presence of lactobacilli and enterococci. A further 38 faecal samples from infants were assessed for bifidobacteria using both bacteriological and FISH methods (Figure 11).

Infants. The prevalence of bifidobacteria in infants according to the bacteriological methods was 7/38 (18%) and for FISH it was 19/38 (50%). There were only 7 samples with concordant positive results and 12 samples without bifidobacteria using both methods. Thus, the concordance between bacteriological methods and FISH was 50%. Consequently, the sensitivity of the bacteriological method for isolation of bifidobacteria was low in our experimental setting.

The probe Lab158 (Table 5) covers both lactobacilli and enterococci which makes the differentiation of these genera difficult. The prevalence of non-differentiable lactobacilli and enterococci was 27/38 (71%) by FISH, with 11 samples being negative. As per bacteriological methods the prevalence of lactobacilli was 20/38 (52%) and enterococci 33/38 (87%). The non-concordance rate was at least 16% with lower sensitivity for FISH.

Table 10. Comparison of counts (CFU $\log^{10}$/per gram of faeces (median-range)) of bifidobacteria, lactobacilli and enterococci in FISH positive and negative samples.

Genera	FISH pos. Bacteriology pos. (median/range)	n	FISH neg. Bacteriology pos. (median/range)	n	p-value
Bifidobacteria	9.9 (7.3–11.2)	7/38	0	0	nd
Lactobacilli	7.3 (3.3–10.4)	13	7.2 (4.4–7.3)	7	p=0.3
Enterococci	9.3 (3.3–10.0)	26	6.1 (4.3–7.2)	7	p<0.01

The bacteriological counts of FISH-positive and FISH-negative lactobacilli were similar, although the highest lactobacilli numbers occured in FISH-positive samples. Similarly, the counts from FISH-positive enterococci were significantly higher than the counts from FISH-negative enterococci samples (Table 10).

To assess which species of lactobacilli were not detectable using FISH we used ITS-PCR to furthe characterise these samples. Lactobacilli were found in 7 FISH-negative samples: *L. paracasei* (5 cases) and *L. plantarum* and *L. delbrueckii* ssp. *delbrueckii*, both in single cases. We propose that there may be a technical problem with molecular detection of FHEL, as seen with these 6 cases. It is possible that the lysozyme treatment was not sufficient for these bacteria and the probe did not reach the DNA. Both lactobacilli and enterococci

are notably resistant to the lysozyme treatment as shown previously (Harmsen, 1999).

Adults. In 24 faecal samples from adults the prevalence of lactobacilli and enterococci using FISH was higher 24/24 (100%) than in the isolates from children (71%). Similarly, when using bacteriological methods the prevalence of lactobacilli 23/24 (96%) and enterococci 24/24 (100%) was higher than that of infants (52% and 87%, respectively). In adults the concordance of results for bacteriological methods and FISH was 96% for both lactobacilli and enterococci. In addition, the counts of LAB were higher using the FISH method for both children and adults (Table 11).

Table 11. The counts of lactobacilli and enterococci in infants and adults using bacteriology and FISH (CFU $\log^{10}$ per gram of faeces; median-range).

Age groups	Bacteriology		FISH
	Lactobacilli	Enterococci	Lactobacilli/Enterococci
Children	7.25 (3.3–10.4)	8.6 (4.3–10)	9.3 (8.0–9.6)
Adults	5.9 (2.6–8.6)	5.9 (3.3–8.08)	8.4 (8.1–9.0)

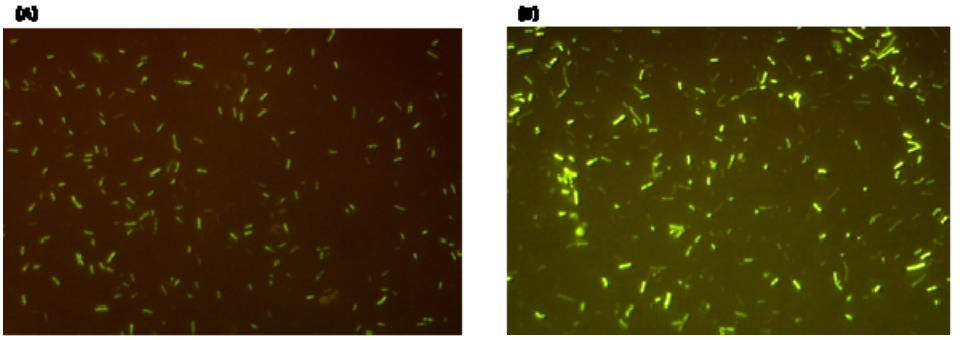


Figure 11. Bifidobacteria (A) and lactobacilli-enterococci (B) in the faecal samples detected with FITC-labeled Bif164 and Lab158 probes.

5.4. Diversity of gut lactobacilli in healthy adults and the elderly (Paper IV)

The counts and species distribution of intestinal lactobacilli in 24 healthy adults and 37 elderly persons was assessed. Lactobacilli were cultured from specimens from 23/24 (96%) adults and 36/37 (97%) elderly people. In two individuals viable counts were below the detection limit. According to RT-qPCR results, lactobacilli were found in all faecal samples of adults and the elderly. Both methods showed that in the elderly persons the total counts of lactobacilli were significantly higher than in the adults: (range 3.6–10.8, median 6,4 vs. range 2.6–8.6, median 5,9; p=0.008) using both the bacteriological method (range

7.0–9.5, median 7,9; vs. range 5.3–8.8, median 7,2; p=0.0002) and using qRT-PCR (Figure 12).

According to the PCR method, the number of species in adults was lower than in the elderly, 4 versus 12 respectively [median 8, species *vs.* 5 to 11 (median 6) (p=0.042)]. There was no correlation between the number of species and the lactobacilli counts, either using bacteriological or RT-PCR methods. The most prevalent *Lactobacillus* species detected in both groups were *L. casei* from the FHEL group and *L. ruminis* from the OHOL group (≥70%; Figure 13). The prevalence of *L. crispatus* was the lowest (8%).

In the FHEL group, *L. plantarum* (22/37, 59% *vs*. 7/24, 29%; p=0.035) and *L*. *paracasei* (36/37, 97% *vs*. 14/24, 58%; p=0.0002) were more prevalent in the elderly people than adults. However, *L. rhamnosus* was detected in 7 (29%) of the adults (p<0.001), but not in any elderly person. The species difference was also found in the OHEL group where the prevalence of *L. reuteri* (19/37, 51% *vs*. 5/24, 21%; p=0.031) and *L. buchneri* (11/37, 29% *vs*. 2/24, 8%; p=0.059), was higher in elderly persons.

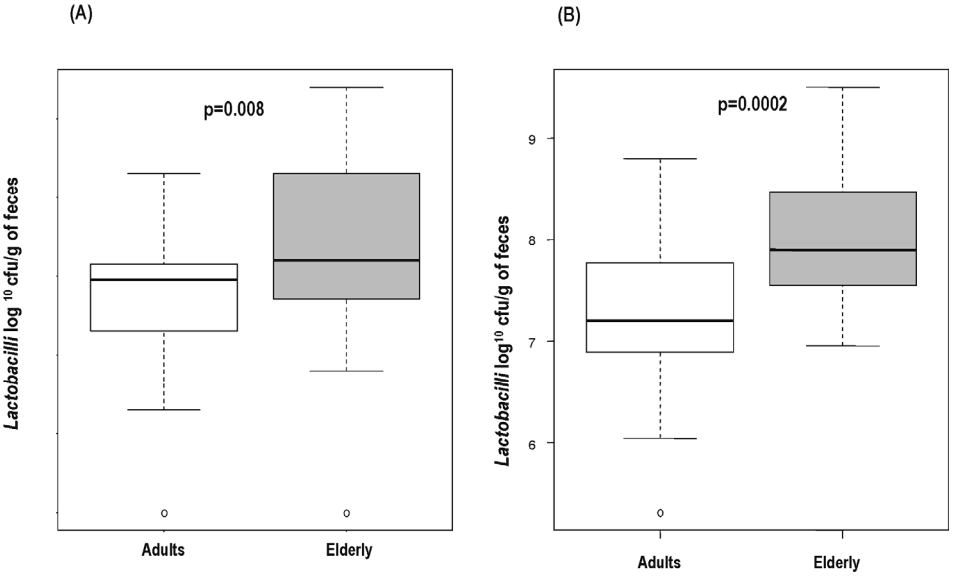
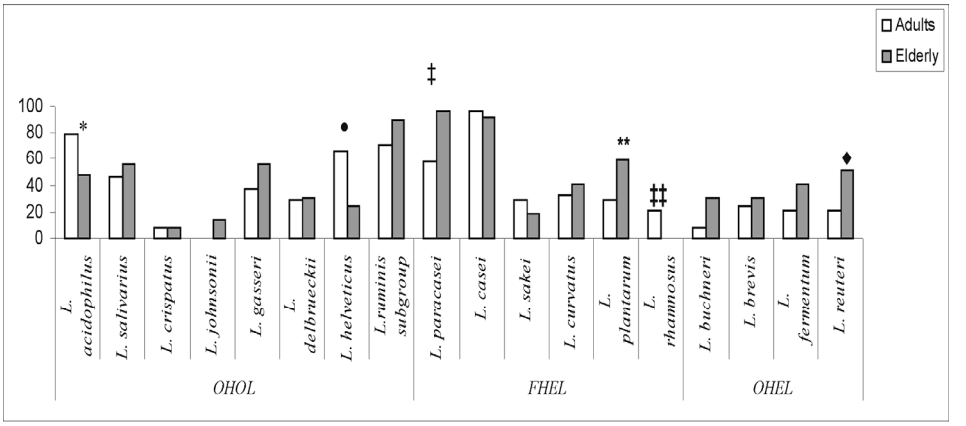


Figure 12. Total counts of *Lactobacillus* spp. in adults and the elderly persons using bacteriology (A) and real-time PCR (B).



*,♦ p=0.031; • p=0.001; ‡ p=0.0002; ** p=0.035; ‡‡ p=0.007

Figure 13. Intestinal *Lactobacillus* species diversity in the adults and the elderly.

5.4.1. Association of *Lactobacillus* spp. with some anthropometrical and metabolic characteristics in healthy adults and elderly (Paper IV)

Age-related, significant differences were found for 6 species of *Lactobacillus*. With regards to the OHOL metabolic pattern, the adults, in comparison to the elderly, were more often colonised with *L. acidophilus* (19/24, 79% *vs*. 18/37, 47%; p=0.031) and *L. helveticus* (16/24, 66% *vs*. 9/37, 24%; p=0.001). *L. johnsonii* was detected only in samples from the elderly (5/37, 13.5%) (Figure 13) and the age range for detection was older than 66, median 68 years old. The age range for detection of *L. rhamnosus*, found only in adults, was 48 with median of 31 years old.

A positive correlation between BMI and the age was found (r=0.301; p=0.0185), though the gender was a confounding factor (Table 12). Furthermore, a positive correlation was found between BMI and fasting blood glucose content (r=0.463; p<0.0001) adjusted for the age and gender. A lower blood glucose level was predicted by the presence of intestinal *L. paracasei* (r^2=0.281; Adj r^2=0.213; p=0.031) with results adjusted for the age in the adult group. A negative correlation of borderline significance was found between the level of blood glucose and colonisation by *L. fermentum* (r=–0.321; p=0.052) in the elderly group. Multiple regression analysis revealed that the BMI was associated with the counts of cultivable lactobacilli adjusted for the age and gender (r^2=0.187; Adj r^2=0.144; p=0.008). The higher BMI in both groups of persons was directly predicted by the presence of OHOL and facultatively heterofermentative *L. sakei* species, both adjusted for the age and gender (Table 12).

Table 12. Spearman rank order correlation, linear, and multiple linear regression analysis between the counts and the species of lactobacilli, BMI, age, and gender.

Variable 1	Variable 2	Correlation coefficients r- and p-values	Linear multiple regression analysis (R^2; Adj R^2; p-values)
BMI (kg/m^2)	Age (years)	r= 0.301; p=0.0185	NS
BMI (kg/m^2)	Fasting plasma glucose (mmol/L)	r= 0.463; p=0.0001	Age, gender R^2=0.182; Adj R^2=0.139; p=0.009
Fasting plasma glucose (mmol/L)	Presence of *L. paracasei*	r= –0.460; p=0.0238	Age (adult group) R^2=0.281; Adj R^2=0.213; p=0.031
Fasting plasma glucose (mmol/L)	Presence of *L. fermentum*	r=–0.321; p=0.052	NS
BMI (kg/m^2)	Counts of lactobacilli (bacteriology; log^{10} CFU/ g of faeces)	r=0.309; p=0.00279	Age, gender R^2=0.187; Adj R^2=0.144; p=0.008
BMI (kg/m^2)	Number of OHOL species	r=0.598; p=0.00213	NS
BMI (kg/m^2)	Presence of *L. sakei*	r= 0.394; p=0.002	Age, gender R^2=0.282; Adj R^2=0.244; p<0.001

NS – no significant difference

5.5. Influence of some environmental factors on gut microbiota

5.5.1. Diversity of gut lactobacilli in 1–2-year-old Estonian and Swedish children (Paper III)

We found that Estonian children were more frequently colonised with lactobacilli than Swedish children (50/71 (70%) *vs*. 30/65 (46%); p<0.01). The distribution of different fermentation types (OHOL, FHEL and OHEL) of lactobacilli was similar in both Estonian and Swedish children with FHEL prevailing in both groups (72% and 80% of colonised children, respectively). The prevalence of intestinal lactobacilli was compared for a set of Estonian (n=18) and Swedish (n=13) 1–2-year-old children. The *Lactobacillus* spp. identified in faecal samples of both groups of children included *L. acidophilus*, *L. paracasei, L. coprophilus, L. delbrueckii*, *L. salivarius*, *L. plantarum* and *L. brevis*. In contrast, *L. fermentum* and *L. buchneri* were detected only in faecal samples of

Estonian children. In addition, *L. plantarum* was identified as the prevailing species for six of the 18 Estonian children tested but none of the 13 Swedish children tested ($p<0.05$) (Figure 14).

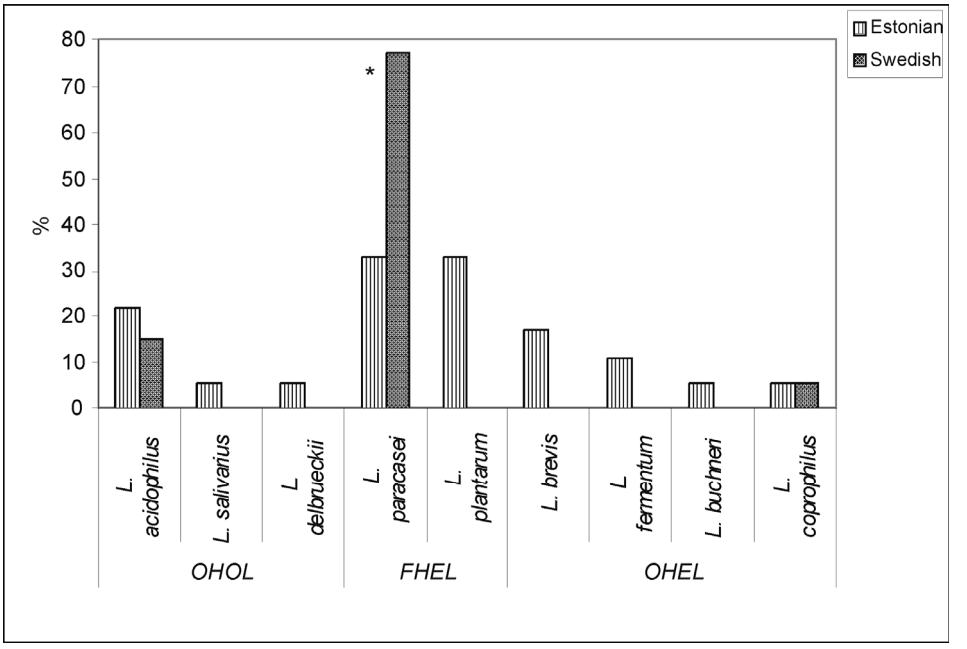


Figure 14. Prevalence of *Lactobacillus* spp. in healthy 1–2-year-old Estonian and Swedish children (*–$p<0.05$).

5.6. The diversity of the bifidobacterial community in non-allergic and allergic children (Paper V)

The prevalence of intestinal bifidobacteria between allergic and non-allergic 5-year-old Estonian children was compared. Bifidobacteria were detected in all samples of allergic and non-allergic children. There were no differences in the prevalence of *Bifidobcterium* spp. between allergic and non-allergic children. Bifidobacterial profiles varied from 3 to 6 dominant bands on DGGE gels (Figure 15).

A total of 128 bifidobacterial clones (55 from allergic and 73 from non-allergic children) were subjected to sequence analysis. The sequence analysis of the 16S rDNA amplicons showed clear difference in composition of the faecal bifidobacterial species in allergic and non-allergic children. Comparison of the obtained sequences with those of the database revealed a significant degree of similarity of the fragments (97–100%) to the 16S rDNA sequences of known *Bifidobacterium* species. *B. adolescentis* was found in the faecal samples of 14 (70%) allergic children and in only 5 (25%) non-allergic children (Figure 16

(A); 14/6 vs. 5/15; OR = 5.8 (95% CI 1.7–19.7); p=0.01) while a significantly higher number of individual clones of *B. adolescentis* was present in allergic children (18/55 vs. 5/73; p<0,001). In contrast, in non-allergic children the *B. catenulatum/pseudo-catenulatum* group prevailed (14/6 vs. 5/15; p=0.01) showing a higher number of different clones as compared to allergic children (23/73 vs. 5/55; p=0.005) (Figure 16 (B)).

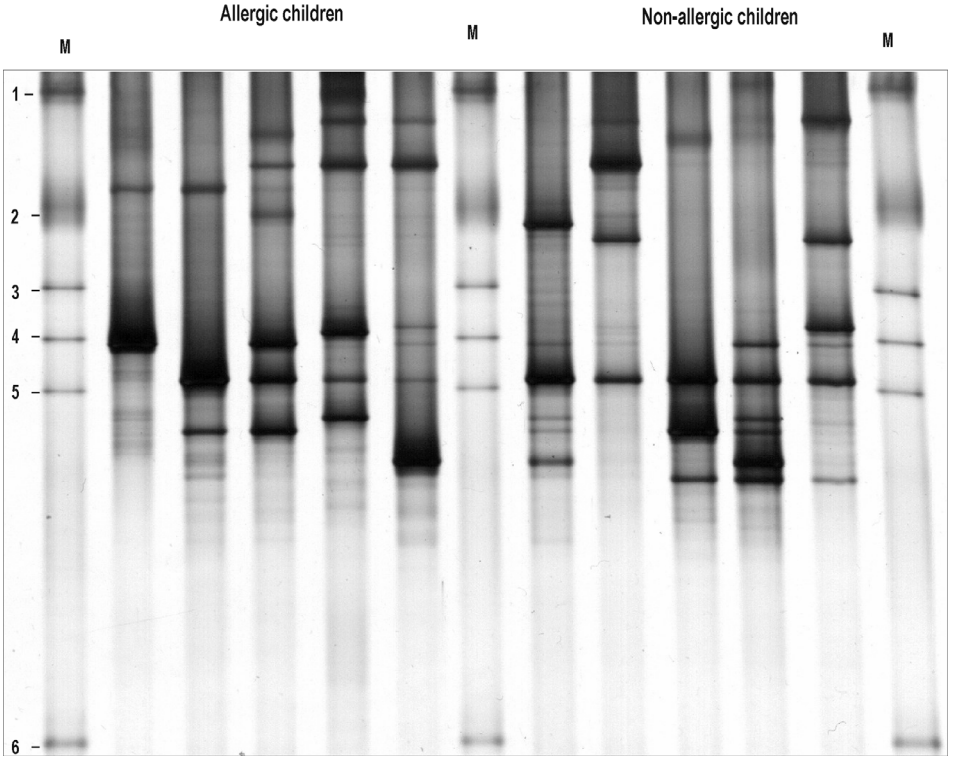


Figure 15. DGGE analysis of the *Bifidobacterium* spp. from fecal samples of allergic and non-allergic children. (M)-indicates the marker for DGGE, constructed from 16S rDNA amplicons: 1 – *Staphylococcus aureus*; 2 – *Enterococcus faecalis*; 3 – *Lactobacillus acidophilus*; 4 – *Escherichia coli*; 5 – *Ruminococcus obeum*; 6 – *Bifidobacterium longum*.

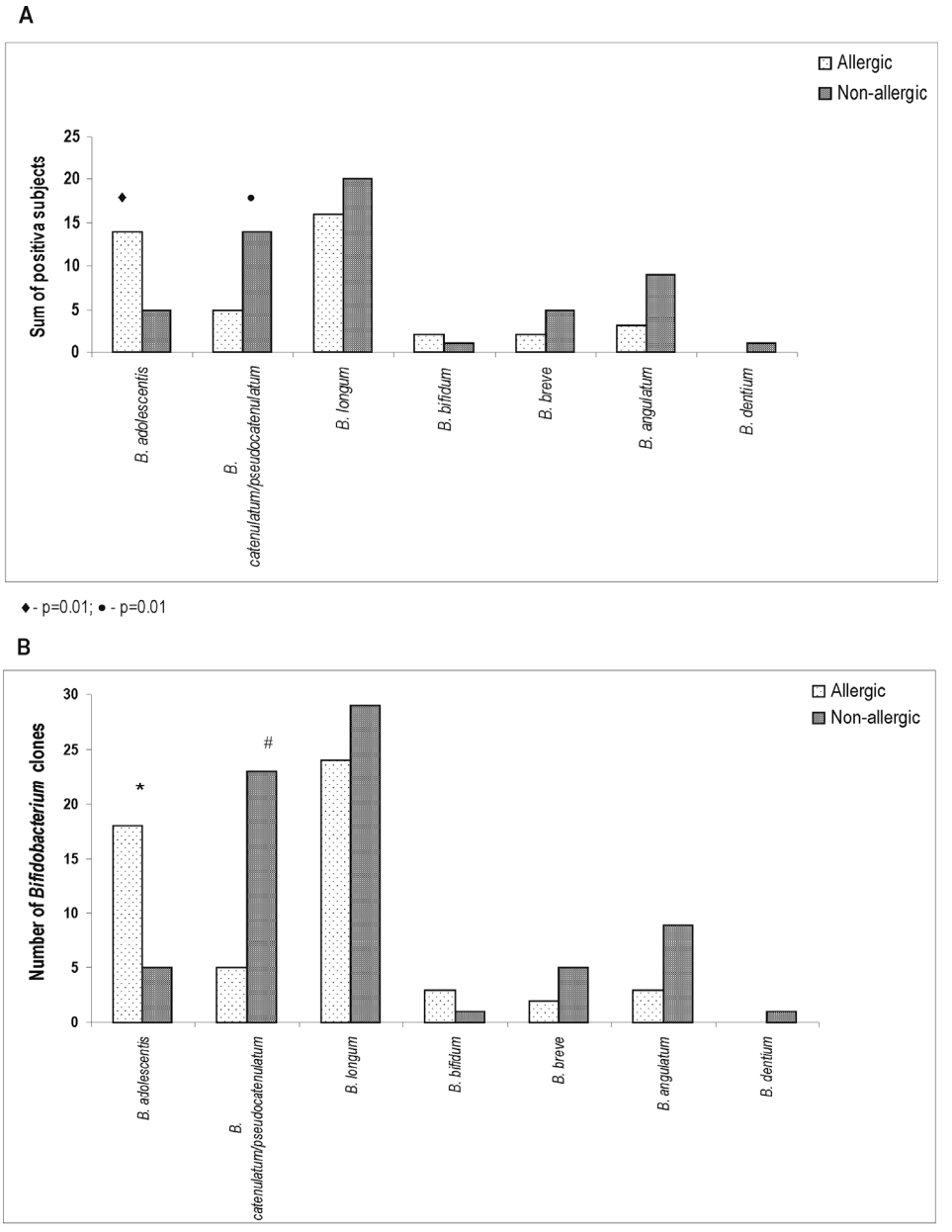


Figure 16. Number of different species (A) and clones (B) of *Bifidobacterium* spp. in allergic and non-allergic children.

6. GENERAL DISCUSSION

Selection of identification and screening methods for functional properties of lactic acid bacteria

The intestinal microbiota is an important factor for human well-being and health. It provides an interface with the environment, particularly involving nutrition for the host and acting as a barrier against potentially harmful food components and pathogenic bacteria. The microbial ecology of the intestine is characteried with the balance between different groups of microbiota in the intestinal lumen and mucosa (Savage, 1978; Mikelsaar, 1993; Zoetendal et al., 2006; Sekirov et al., 2010; Cerf-Bensussan et al., 2010). Many different diseases and also their prevention can be linked to a dysfunctional intestinal microbiota. It is obvious that understanding the cross-talk that occurs between intestinal microbiota and its host promises to expand our views about the relationship between intestinal microbiota and health. The recognition of LAB as a part of the human microbial ecosystem and the understanding of various interconnected influences of that system is an important step for their application as healthy functional food (Tannock, 2004; Vaughan et al., 2005; Walter, 2008). There is still much to be learned about the normal LAB microbiota and how to achieve a desirable composition. Moreover, the precise identification of these bacteria at the species level is not an easy task.

Prevalence, estimation and enumeration of LAB by molecular methods

In the present thesis, different methods for chracterisation of phenotypic and genetic properties of LAB intestinal isolates were compared. In particular, the human LAB microbiota from different age groups, geographical locations and health status were examined utilising methods fordifferentiation based on phenotypic and molecular characterisitcs as laid out in the stated aims.

Previously, several methods have been used for quantitative and qualitative assessment of intestinal LAB, with a wide variation in results between the various authors. Our particular interest concerned both bifidobacteria and lactobacilli. Bifidobacteria have been described as strictly anaerobic bacteria. Identification of anaerobic bacteria often requires very laborious, complex and time-consuming methods in addition to specifically developed anaerobic tehniques (Langendijk et al., 1995). Previous work in this field has been greatly hampered by the methodical inaccuracy that comes with handling these bacterial cultures in anaerobiosis. Many bacteria are fastidious in nature or are unculturable using current methods. Furthermore, media are frequently non-specific or are too selective for certain bacteria.

The main focus for quantitative and qualitative identification of microbes has moved from phenotypical to genotypical methods as the latter has generated more sensitive and accurate results (Lick et al., 2003). Molecular techniques for

quantitative microbial community analysis that do not require isolation of the microorganisms are very promising for providing an overview of the diversity of microorganisms present in particular samples or ecosystems. Without doubt, one of the most direct methods for the identification of single cells within a complex ecosystem is using FISH with specific 16S r-RNA-based oligonucletide probes (Amman et al., 1995; Langendijk et al., 1995; Harmsen et al., 1999). Approximately 90% of the total faecal bacteria can be detected using FISH with different group-specific probes. However, according to the literature the sensitivity of FISH based on microscopy is not high. The detection limit for bacteria is 10^7–10^8 cells per gram of faeces against a total population background of 10^{11} cells or just 0.1% of the total population. The quantitative information derived from the FISH method and the qualitative information derived from other PCR-based methods may be complemented by more advanced quantitative PCR to obtain results which may reflect the real situation within this ecosystem.

In our study, the counts of intestinal lactobacilli from different age groups of people were some 10–100 times higher when molecular methods were used such as FISH and RT-PCR when compared to attempts to cultre these on media. This corresponds to the data of Eckburg et al. (2005) where molecular analysis showed that facultative anaerobic bacteria were only a minority within the colon (0.1%) and that strictly anaerobic microbes were by far the most dominant group of microorganisms. FISH has been relatively widely used for intestinal microbiota assessment and it has also been used to assess changes in levels of the predominant groups of intestinal bacteria as a result of the consumpsion of prebiotic substances or probiotic bacteria (Kirjavainen et al., 2002; Tuohy et al., 2003). Interestingly, using FISH it has also been shown that there are some differences in the gut microbiota between infants who later develop atopy and those who do not (Kalliomäki et al., 2001).

In our study we have used selective media for the simultaneous detection of bifidobacteria (CAN; BBL) and lactobacilli (MRS). The low corrsepondence between cultural and FISH methodologies, the latter using the Bif164 probe illustrated a methodical failure in the cultivation of bifidobacteria. Discrepancy between bacteriological and FISH methods may be related to the ability of bifidobacteria to grow on non-specific medium. The ability of bifidobacteria to grow on MRS medium is poor (Chapin et al., 1999). In contrast, Harmsen showed that 90% of colonies grown on media selective for lactobacilli, specifically the MRS and Rogosa plates, were hybridised using the Bif164 probe and not with the Lab158 probe. They concluded that species such as *Bifidobacterium* spp., which are dominant in the samples, grew also on the MRS selective media (Harmsen et al., 2000). However, in our study, all the isolates of the gram-positive rods from MRS media were identified as *L. paracasei, L. plantarum, L. delbrueckii* ssp. *delbrueckii* and no unidentified isolates (putatively bifidobacteria) were revealed. Our results were not affected by sample storage problems as in our study even more positive samples were detected using arti-

ficial culture media than molecular typing. This implies isolation problems exist with the strictly anaerobic bifidobacteria. This is in agreement with previous reports which showed that *B. adolescentis* could be better identified using PCR-DGGE, sequencing and FISH methods than with cultivation techniques (Matsuki et al., 1999; Welling et al., 1999). Moreover, a recent study using FISH combined with flow cytometry has confirmed that *B. adolescentis*-related species are likely to be dead or damaged by the time faecal samples are passed to the laboratory (Ben-Amor et al., 2005).

Simultaneously, issues have arisen using the Lab158 probe which detects enterococci-lactobacilli phyla. Using FISH faecal enterococci were frequently misidentified for lactobacilli, since the cells of both bacteria may show as plump rods. Furthermore, in some FISH-negative samples low counts of enterococci were detected on culture media which were seemingly missed using the molecular analysis. In general, probes containing a single labeled molecule give a strong signal only if cells are metabolically active and, hence, contain large numbers of ribosomes and target rRNA (Hahn et al., 1992; Manz et al., 1993). A limitation of FISH may include problems with cell wall permeability (Bindenko et al., 1998) which gives rise to difference in the abilities of the probes to reach target sites (Fuchs et al., 1998). In the current study we used lysozyme to resolve the problems with cell wall permeability. Low fluorescence levels in positively hybridized cells can also significantly overlap with high background signals from negative controls (Langendijk et al., 1995). Coaggregation of bacteria, broken cells, or contaminating substance can make cell identification and counting even more difficult. It has been reported that for estimation of *Bifidobacterium* spp., molecular methods such as FISH and PCR-DGGE method combined with sequencing is a more appropriate approach. The use of DGGE/TGGE profiles may represent 90–99% of the bacterial community, with the detection limit in faecal samples at 10^9 cells per gram of faeces (Zoetendal et al., 1998). This particular approach was successfully applied byour group in this present study to analyse the species composition of bifidobacteria in 5-year-old allergic children.

However, even the latest molecular approaches have several limitations, including the extraction of nucleic acids from samples, biases, and artefacts associated with enzymatic amplification of the nucleic acids, cloning of PCR products, as well as the previously described sensitivity and target site accessibility in whole-cell hybridization techniques (Head et al., 1998). Futhermore, as more rRNA sequences become available in sequence databases, it has been highlighted that may probes and primers do not respond to the taxonomic level desired. To date we are still far from having all the necessary specific primers and probes for LAB species and questions arise regarding the specificity and validity of existing ones which should be checked systematically with close genera, species and strains.

Historically of course, culture methods have been extensively used as the gold standard to quantitate LAB in faeces. However, it has been suggested that

the contrasting results in various studies seen may be due to the high variability of the intestinal microbiota between individuals in addition to the variability seen in the methods used, such as different culture media for the counting or isolation of strains or different methodologies used for identification of isolates. Conventional culture-dependent methods have limitations such as low sensitivity, long duration, as well as bias introduced due to the culture, and recovery of only the culturable species of the intestinal microbiota. Culture-based analysis may, however, give qualitative and quantitative fluctuations especially in the faecal microbiota compared to molecular methodology. This can lead to the overestimation of some species and underestimation of others.

However, culture-based methods have some advantages. We have found that using culture-based techniques when applied to the analysis of intestinal microbiota, several new associations with human health indices can be detected. Thus, the bacteriological methods may provide a complementary picture of the LAB populations in humans.

Identification methods of intestinal *Lactobacillus* isolates: phenotypic API 50CHL *vs.* molecular (ITS-PCR) methods

Though molecular methods have brought new aspects to the phylogenetic and quantitative relations of different bacteria in the gut, culture-based and molecular-based profiling may be considered complementary with both giving a broadly similar overview of the intestinal ecosystem.

Frequently in the identification and species differnetiation of lactobacilli isolates methods have been based on their phenotypic properties, particularly the fermentation patterns of carbohydrates. The API 50CHL test was developed for the identification of human *Lactobacillus* strains in 1980s. However, its reliability is in question because the applied taxonomy of the genus *Lactobacillus* is unsatisfactory due to phenotypic heterogenecity (Andrighetto et al., 1998). The ITS-PCR method is a highly discriminatory molecular typing technique for lactobacilli, which can give good differentiation at the strain level, thus allowing for a comprehensive assessment of the diversity of isolates recovered on artificial media.

In this study, we identified the reference strains and faecal isolates of lactobacilli from different fermentation groups (OHOL, FHEL and OHEL) by using phenotyping (API 50CHL) and compared these results to molecular (ITS-PCR) methods.

According to the results using the API 50CHL test, in several cases, the exact identifications of closely related species was not reliable; some were doubtful or unacceptable and some strains were misidentified despite a stated "good" identification level. Differences in identification were found by both methods in all groups; especially for the *L. acidophilus* group. We found that the API system assigned to some species such as *L. gasseri*, *L. johnsonii*, *L. jensenii*, *L. ruminis* and *L. helveticus* for *L. acidophilus*. It may be concluded

that sometimes the low fermentation ability of carbohydrates is not sensitive enough for differentiation of species among the entire OHOL group. Also the manufacturer's database may not be up to date in this dynamic area and that profiles for some prevalent *Lactobacillus* spp. of the OHOL group may be deficient. Moreover, 16S rRNA sequence analysis for *Lactobacillus* spp. has shown that some phenotypically generated taxa do not correspond with the suggested phylogenetic relations in particular in the case of *L. acidophilus* and *L. paracasei* groups (Holzapfel et al., 2001).

Such differences were also seen in the FHEL and OHEL groups. Within the FHEL group, we found that lactobacilli isolates identified by phenotyping such as *L. rhamnosus*, *L. curvatus* were reassigned using ITS-PCR as *L. paracasei* ssp. *paracasei*. Similarly to Tynkkynen et al. (1999) our study showed when using API 50CHL for identifying the strains of the *L. casei* group (*L. rhamnosus*, *L. paracasei*, *L. casei* and *L. zeae*) several reassignments were necessary based on molecular identification.

In our study, the species differentiation among OHEL group, including *L. fermentum*, *L. cellobiosus*, *L. reuteri* and *L. buchneri* was problematic using phenotypic typing methods. In addition, no genotypic differences were noted between *L. fermentum* and *L. cellobiosus*. It has been revealed by DNA/DNA hybridization that *L. cellobiosus* is identical to *L. fermentum* (Vescovo et al., 1979) and the first name is now considered as synonym to the latter (Hammes et al., 1992). In the study of Dellaglio et al. (2004) phylogenetic placement of *L. cellobiosus* was obtained based on 16S rDNA sequences, and genetic similarity was investigated by comparing partial *recA* gene sequences for the type strains of *L. cellobiosus* and *L. fermentum*. Based on the high identity values for 16S rDNA (99%) and recA gene (98%) sequences, the results of DNA–DNA hybridization assays and phenotypic traits available from the literature, it is proposed that *L. cellobiosus* be reclassified and, as a rule of priority, renamed *as L. fermentum*.

Thus, in conclusion, the present investigation highlighted a discrepancy between API 50CHL based phenotypic and genotypic identification techniques and suggested that API 50CHL analysis be used only as a preliminary identification tool. This is in agreement with proposals by other researchers in this field (Nigatu, 2000; Paludan-Müller et al., 2002; Lei, 2004). However, it should be borne in mind that API 50CHL analysis provides important data regarding the carbohydrate fermentation patterns of the isolates, which are important from a functional and applied point of view (Sawadogo-Lingani et al., 2010).

Lactoflora in children, adults and elderly

In our study faecal *Lactobacillus* populations were was studied in healthy children, adults and also in the elderly. We focussed on *Lactobacillus* species which although they comprise only a minor part of the bacterial community in human

faeces (Sghir et al., 2000) is more prevalent in the caecum and the physiologically important area of the ileum (Marteau et al., 2001).

Previous culture-based studies have indicated higher viable counts of lactobacilli in healthy elderly people as compared to adults (Mikelsaar and Mändar, 1993). In our study, lactobacilli were detected in 90% of faecal samples using bacteriological methods and in all samples by RT-PCR. In the elderly, higher counts of intestinal lactobacilli were assessed by both methodical approaches. In addition, other data, based on viable counts, has shown that lactobacilli are present in the gastrointestinal tract of 70–73% of a population which consumes a Western-type diet whereas within those consuming a vegetarian diet a higher prevalence of lactobacilli has been assessed (Finegold et al., 1977,1983; Tannock, 1991). In Estonia, Western-type diet features have been attributed with the high use of fiber-rich and dairy products that could be the cause for differences.

The *Lactobacillus* species composition of the lower parts of the gastrointestinal tract is still not well described. Moroever, the composition of intestinal lactobacilli also varies widely between individuals. Our results confirm that each person from the different age groups analysed had an individual pattern (from 4 to 12 species) of different lactobacilli species, which is in agreement with previous studies (Ahrne et. al., 1998; Delgado et al., 2007). Present knowledge indicates that 20 *Lactobacillus* species are putative inhabitants of the human gut, some of which are only recently detected using molecular methods with specific primers for LAB. From 20 lactobacilli species identified in this study, 18 were previously described as members of faecal microbiota of humans, although some have only been detected by molecular techniques using specific PCR primers. These include species such as *L. gasseri* and *L. ruminis* group. In the present study, *L. acidophilus*, *L. salivarius, L. paracasei*, *L. casei, L. plantarum, L. brevis* and *L. fermentum* were the most common species in all age groups, which is in agreement with previous studies. However, in our study we also found age-related differences in species profile. With regard to *Lactobacillus* species distribution between age groups, we found that *L. paracasei* and *L. plantarum* dominated in 1–2-year-old children and elderly persons. It has been previously reported that from 12 months, *L. paracasei* starts to be the most prevalent *Lactobacillus* species, and it becomes more prominent at 18 months, followed by *L. delbrueckii*, *L. plantarum* and *L. acidophilus* (Ahrne et al., 2005). *L. gasseri* and *L. fermentum* were also detected in faecal samples of children, which agree with the data of Vassos (2007).

Among the faecal lactobacilli of adults, *L. acidophilus* and *L. helveticus* were the most commonly detected habitants, being present in more than 60% of the individuals. Early studies based on bacteriological methods identified *L. acidophilus* as the predominant faecal *Lactobacillus* species in adults (Hentges et al., 1993; Moore et al., 1995). On the basis of the current taxonomy and using molecular methods, most of the *L. acidophilus* isolates were classified as *L. gasseri, L. helveticus* and *L. crispatus* in our study. Thus, isolates from

L. acidophilus group are difficult to distinguish, and it may lead to inaccurate results.

In our study, *L. ruminis* and *L. casei* were also detected as common species in the *Lactobacillus* biota of adults and the elderly as has been previously reported (Vaughan et al., 2005).

L. sakei and *L. curvatus* are food-associated lactobacilli and these could be detected by direct analysis of the 16S rRNA or 16–23S rRNA genes but not by bacteriological culture (Walter et al., 2001; Heilig et al., 2002).

A striking difference was noted concerning the *L. rhamnosus* species, which could be found in faecal samples of children and adults, but not in the elderly. *L. rhamnosus* has not been found to predominate studies of infants from the different countries such as the Netherlads, Japan and Scotland (Song et al., 1999; Harmsen et al., 2000; Satokari et al., 2002). However, it has been shown to be common in a study of Greek infants (Mitsou et al., 2008). Walter (2008) has suggested that *L. rhamnosus* is one of the dominant *Lactobacillus* spp., though only 26% of the healthy individuals and 6% of the elderly have been shown to harbour this species (Ahrne et al., 1998; Silvi et al., 2003). But why *L. rhamnosus* is completely absent in the elderly according to our data is not clear. Interestingly, Gefilus, one of the *L. rhamnosus* GG products in Estonia, is heavily marketed as a probiotic. We hypothesize that in the infancy of the cohort of elderly people studied (born between 1920–1940) when the resident *Lactobacillus* spp. biota of gastrointestinal tract were formed, the diet and environment did not favor the colonisation of gut with this species. Further, during the following years of life, even the intensive spread of this probiotic strain did not help to colonise this cohort which had a well-formed microbiota.

Reuter and coauthors (2001) have observed the predominance of *L. reuteri* among the indigenous intestinal lactobacilli in contrast to other authors who reported this species as only a minor component of the obligately heterofermentative group or completely absent in humans (Vaughan et al., 2005). In our investigations, altogether 39% of humans harboured *L. reuteri.*

A few studies have indicated that *Lactobacillus* populations in the human gut show temporal dynamics characterised by species and strain fluctuations (Mueller et al., 2006; Vassos, 2007). However, several authors (Mitsuoka et al., 1992; Reuter, 2001; Tannock et al., 2002) have identified the following as persistent strains in intestinal tract; *L. gasseri, L. crispatus, L. reuteri, L. salivarius* and *L. ruminis* and these were detected also in our study. However, seemingly in some persons the *Lactobacillus* spp. such as *L. acidophilus, L. casei, L. paracasei, L. rhamnosus, L. delbrueckii, L. brevis, L. johnsonii, L. plantarum* and *L. fermentum* still perform stable populations of the gut. The persistent colonisation of intestines with *L. acidophilus, L. fermentum* and *L. casei* has been shown in healthy persons over a 15 years period (Mikelsaar et al., 1998) in opposite to suggestion by (Walter, 2008).

Lactic acid bacteria metabolites and their association with some health indices of the human body

While a first step in studying LAB in the GI tract is the identification of the microbes, a subsequent step is identifying the role of LAB metabolites in the health of its human host. Convincing evidence of the metabolic impact of lactic acid bacteria on their host remains deficient. It is not yet clear as to whether different fermentative groups and species of lactobacilli with different metabolic endproducts affect differentially the anthropometrical and biochemical indices of humans.

Phospholipid contents of bacteria vary across taxa, and fatty acid profiles of individual bacteria are known to vary quantitatively and qualitatively with changes in environmental factors (Rose, 1989). Cell fatty composition is especially sensitive to medium composition, but also incubation temperature, cell age, and degree of anaerobiosis as occurs within the intestines (Moore, 1993). In our study we found that production of organic acids by LAB depends on environmental conditions.

There is considerable evidence that individual SCFA may have specific roles in connection with health implications. Furthermore, it is well known that SCFA and lactic acid stimulate gastric motility and gastric absorption, affect pancreatic secretion, promote trophics of intestinal mucosa and affect the proliferation of pathogenic bacteria in gastrointestinal tract. It has been previously described that lactic acid has a higher antimicrobial activity than acetic acid (Ouwehand, 1998). This activity may also be affected by the synergistic activity of different acids (Ouwehand, 1998). Thus, the test milieu, e.g. a micro- or anaerobic environment, seems to be very important for the expression of antimicrobial metabolites and subsequent selection of strains.

Microbes sometimes influence host physiology in a way what is not directly related to the GI tract function (Zoetendal et al., 2006). The principal substrate available for microbial fermentation in the large intestine includes a wide variety of dietary residues that have escaped digestion in the small intestine, the main ones being non-starch polysaccharides (NSP), various forms of starch resistant to digestion in the small intestine, sugar alcohols and proteins. Also, host-produced substrates such as glycoproteins of exfoliated epithelial cells and pancreatic secretions are other important sources. In particular, NSP are important energy substrates for large intestinal microbial fermentation, and the amount as well as the chemical and structural composition of the carbohydrate, are all important factors for the microbial activity within the gastrointestinal tract (Jensen and Jorgensen, 1994; Jensen, 2001). The energy available to the host after microbial fermentation is the energy found as SCFA, based on approximately 60% of that of the substrate being fermented (Jensen, 2001).

Whether these differences are also expressed in the different habitat of the gut (small and large intestine) with different oxygen tensions, has to be elucidated by GC testing of human samples from different parts of GI tract. The

intestinal lactobacilli can support intestinal epithelial cell proliferation through the production of SCFA which provides energy for the host (Shanahan, 2002). For example the main fermentation end product of OHOL group is lactic acid. In contrast, lactobacilli of the FHEL and OHEL groups, including *L. paracasei* and *L. fermentum,* play an important role in carbohydrate breakdown producing lactic acid, acetic acid and also succinate. It can be speculated that intestinal colonisation by some species of the latter groups decreases the content of both glucose (hexose) and fructose (pentose) due the effecient phosphoketolase pathway of carbohydrate fermentation (Axelsson, 1998). Thus, the complex metabolic activities between host and different groups of microbiota are capable of shaping the nutrient environment of the gut and subsequently the blood glucose level (Falk et al., 1998).

Previously, it has been shown that the elderly are more prone to metabolic syndrome, expressing a higher blood glucose level as part of this. To date, overweight children and adults also show an increased trend to develop a metabolic syndrome. A possible connection between the prevalence of *Firmicutes* and fat storage has been demonstrated (Bäckhed et al., 2004). Through the production of SCFA, the intestinal lactobacilli may positively influence intestinal epithelial cell proliferation and provide energy for the host.

We have shown that a reduction in oxidative stress indices (oxidized low density lipid content) was associated with higher numbers of lactobacilli in the elderly (Mikelsaar et al., 2010). Furthermore, we have raised the question as to whether the high counts of lactobacilli and prevalence of different species, particularly those of different fermentation groups, could influence the health parameters such as blood glucose levels and BMI. According to our study, isolation of *L. sakei* was associated with a higher BMI. The ability of this species to metabolise complex carbohydrates to monosaccharides has been shown previously and may also support their absorption and harvesting by host cells. Some studies indicate that *L. sakei* is one of the predominant food-associated *Lactobacillus* species that occurs in human faeces (Hammes et al., 1990, 1998). This may be explained by the frequent occurrence of this species in the food environment. *L. sakei* has been isolated from meat, sausages, and sauerkrauts and is one of the major spoilage organisms for vacuum-packaged meat products. In addition, it is an important component of starter cultures used for the production of fermented meat products.

Interestingly we found a reduced blood glucose level in adults colonised with *L. paracasei* from the FHEL group. This finding is supported by the data of Matzuzaki and coauthors (1998), which have shown that administration of the closely related *L. casei* to a diabetic mouse model, reduced blood glucose levels. Likewise, a diet enriched with another *Lactobacillus* species of the FHEL group (e.g. *L. rhamnosus* GG) has resulted in an improved glucose-tolerance test as well as in reduced blood glycated haemoglobin values in experimental rats and pregnant women (Yadav et al., 2007; Laitinen et al., 2009). Collectively, this data clearly hints at the potential for a positive impact

of probiotics using particular *Lactobacillus* species from different fermentative groups with different effects on the host metabolism.

In addition to SCFA, polyamines play an essential role in the maintenance and enhancement of the mucosal barrier. To illustrate, putrescine is produced straight from ornithine, arginine, is primarily converted into agmantine which is then converted into putrescine (Halaris et al., 2007). Diamine oxidase metabolises putrescine into gamma-butyric, which is an essential molecule in the metabolism of neurons.

In addition, arginine serves as the source of nitric oxide (NO) produced from nitric oxide synthase isofroms which provides a hyperemia-dependent protection mechanism against stress-induced damage of the intestinal mucosa (Brzozowski et al., 2008). Putrescine is produced by several anaerobes, as well as *Escherichia coli* and lactobacilli with lactobacilli comprising a majority of the microflora of the proximal colon (Marteau et al., 2001). Lactobacilli produce polyamines through decarboxylation of amino acids, particularly at the high pH of the intestinal content (Lonvaud-Funel et al., 2001). On the other hand, strains of *Lactobacillus acidophilus* utilise putrescine and reduce the odour of faeces (WO 2008/019887, BASF AG).

Polyamines are produced endogenously or they are obtained actively from food (Stanton et al., 2006). In the case of the damage of epithelial cells, the production of polyamines by the intestinal microflora is considered one of the compensatory mechanisms for modification of immune response and apoptosis regulation.

It is especially important for elderly, whose polyamine-synthesizing ability is lowered, to consume food fortified with polyamines. Only 20% of consumed putrescine is absorbed from intestine into bloodstream, accompanied by a rise of acetylated putrescine while the majority is metabolised within hepatocytes and enterocytes (Milovic et al., 1997).

However, tha ability to perform some biochemical reaction *in vitro* will not always be reflected in host. Phenotypic methods for assessment of the LAB composition and metabolic as well as enzymatic activities of microbial LAB communities in the gut have shown certain inherent limitations but also useful features of the methods used. We have succeeded in showing that polyamine production *in vitro*, within cheese by a strain of *L. plantarum* Inducia was in good correlation with the increased amount of metabolised acetylated putrescine in host urine after consumption of the Inducia cheese.

On the other hand, the high amounts of polyamines could facilitate the absorption of glucose, increasing the number of glucose carriers in the membrane of enterocytes. In Finnish elderly people, it was found recently that there is a positive correlation between the counts of administered *L. acidophilus* NCFM (OHOL group) and the values of polyamine spermine. This may be the reason for an increased BMI tightly bound to the glucose level of blood in persons colonised predominantly with several species from the OHOL group in our study.

Changes of lactic acid bacteria composition by different environmental factors and diseases

Geographical differences

Our results demonstrated that certain regional-specific differences exist in the prevalence of colonisation with particular lactobacilli. Swedish children were less frequently colonised with lactobacilli than Estonian children. However, no differences were observed in the prevalence of different fermentation types among the prevailing *Lactobacillus* isolates in colonised children from either group, indicating a gross uniformity of intestinal lactobacilli profiles between the two populations. FHEL group was dominant in both countries and OHOL and OHEL were equally common among the Estonian and Swedish children. This is in agreement with geographical differences in microbiota species composition which have been described previously (Mueller et al., 2006; Dicksved et al., 2007). In our study, *L. plantarum* strains were encountered only from Estonian children. One explanation for this difference may involve genetic and environmental differences in the human population between the two countries, as the implantation and the development of *Lactobacillus* in the neonate are greatly influenced by prematurity, method of birth, early feeding style (breast milk or formula fed), diet and environment (Fanaro et al., 2003). For example, in adults the prevalence of *L. plantarum* has been associated with a vegetarian as opposed to a typical Western type of diet (Stiles and Holzapfel, 1997). The Estonian diet to a large extent based on locally produced foods and many foodstuffs are fermented by lactic acid bacteria which are a prominent part of the diet, even in children. The individual specificity on the one hand and the temporal modulation of intestinal microbiota by diet, lifestyle, and stress on the other hand complicate the understanding of the dynamic relationship between colonic microbiota signatures and host functions throughout the lifespan (Nielsen et al., 2003; Lay et al., 2005; Guarner, 2006).

Bifidobacterium spp. and allergy

Differences in the *Bifidobacterium* population between allergic and non-allergic infants have been commonly found in previous studies (Bjorksten et al., 1999; 2001; Kalliomaki et al., 2001; Suzuki et al., 2007), in which lower numbers and lower prevalence of *Bifidobacterium* in allergic infants were observed. Also, inadequate bifidobacteria composition in GI tract microbiota has been suggested to be a risk factor for the development of allergy. Watanabe et al. in 2003 showed that the counts of *Bifidobacterium* spp. were significantly lower in patients with atopic dermatitis than in healthy control subjects. In particular, percentages of *Bifidobacterium* were significantly lower in patients with severe skin symptoms than in those with mild skin symptoms. In the previous studies performed in our laboratory using bacteriological methods we could not see any differences in prevalence of *Bifidobacterium* spp. between allergic and healthy

children at the age of 5 (Sepp et al., 2005) However, using specific *Bifidobacterium* primers we observed significant differences in the distribution of *Bifidobacterium* species: allergic children were mainly colonised with *B. adolescentis,* whereas in non-allergic children the *B. catenulatum/pseudocatenulatum* group predominated. These data correspond well to the results reported by Ouwehand and coworkers (2001) who showed using bacteriological methods that 50% of the total bifidobacterial isolates of allergic children were *B. adolescentis*. The culture-independent methods used have shown that the *B. pseudocatenulam/catenulatum* group was the most common species in the adult intestinal tract, followed by *B. longum, B. adolescentis* and *B. bifidum,* whereas *B. breve, B. infantis* and *B. longum* are the predominant species in infants (Matsuki et al., 1999, 2004). In contrast, Gore et al. (2008) showed that *B. pseudocatenulatum* is associated with atopic eczema in infants.

It is difficult to explain why at the age of five years the species composition of bifidobacteria in allergic children differs from healthy children of the same age and also from microbiota of adult persons. It may be that different lactic acid bacteria can modulate the immune response of the host by increasing the IgA synthesis and by inducing different pro-and anti-inflammatory cytokines. The species *B. adolescentis* prevailing in allergic children is more effective at triggering the pro-inflammatory cytokines TNF-α, IL-6 and IL-12, but it is not able to induce the regulatory cytokine IL-10, which is an important anti-allergic immune response (Kramer et al., 1995; He et al., 2002). In contrast, some other species such as *B. bifidum, B. longum* and *B. pseudocatenulatum/catenulatum* induce the production of the regulatory cytokine IL-10 (Young et al., 2004).

Several studies have indicated that supplementation of the child's diet with probiotic lactobacilli and bifidobacteria, such as *Lactobacillus* GG and *Bifidobacterium lactis* Bb-12, can somewhat reduce the allergic disorders in atopic children and may have a strong impact on development, microbial cross-talk, evolution and modulation of a microbiota (Kirjavainen et al., 2002; Gueimonde et al., 2006). It is therefore tempting to suggest that in order to ameliorate allergic disease; the consumption of some probiotics including *B. pseudocatenulateum/catenulatum* may be beneficial.

Characterisation of the probiotic strain, *L. plantarum* Inducia DSM 21379

To characterise our probiotic strain we looked at its molecular profile and its metabolic functional properties. The probiotic capacities are strain-dependent; therefore the methods for reliably identifying LAB at the strain level are of great importance. In the present study the 16S rDNA of the potential probiotic strain *L. plantarum* Inducia DSM 21379 has been sequenced. The sequencing method based on 16S or 23S rDNA is currently considered to be the most powerful and accurate method for determining the degree to which microorganisms are phylogenetically related.

The application of PCR-DGGE and PFGE methods is also valuable, especially for the quality control of approved *Lactobacillus* spp. strains, to avoid

health risks and misleading claims, as well as for the description of new strains. According to our PFGE results, the patented strain *L. plantarum* Inducia DSM 21379 showed clear difference from the other *L. plantarum* strains including the patentable *L. plantarum* Tensia DSM 21380 strain.

Among the functional properties of the Inducia strain the ability to produce putrescine in relatively high amounts was remarkable. Previously, the production of biogenic amines has been most extensively studied with respect to histamine and tyramine, probably the two most important BA of bacterial origin in food, due to their toxic effects. In our study we investigated the ability of some lactobacilli species for their production of bio- and polyamines such as putrescine and cadaverine. Lactobacilli produce polyamines through decarboxlation of amino acids, particularly at the high pH of the intestinal content (Lonvaud-Funel, 2001). Bover-Cid et al. (1999) has shown that 40% of LAB strains were found to be tyramine formers. Only a few strains of lactobacilli produced putrescine and small amount of cadaverine. Several BA-forming strains are of importance in food fermentation. Our study noted that minimal amounts of the biogenic amines were found during *in vitro* tests with *L. plantarum*, and that also minimal amounts were found in cheese comprising *L. plantarum* Inducia and urine samples of *L. plantarum* Inducia consumers. In contrast, the ability of *L. plantarum* strains to produce high levels of putrescine was refleted in urine samples from consumers of this strain. The results of BA production suggest that the capability to produce amines might be strain-dependent rather that being related to specific species. The composition of decarboxylase medium, the incubation time and the particular activity of strains within the cheese matrix and intestinal content play important roles in the production of polyamines by bacterial strains.

CONCLUSIONS

We applied phenotypic and genotypic methods to characterise the composition and the metabolic impact of intestinal lactic acid bacteria and to examine their relationship with different age groups, their environment and atopy.

1. The phenotypic methods used including culture on artifical media with subsequent biochemical profiling by API 50CHL and GC estimation of organic acids and ethanol are appropriate for estimation of the comparative *Lactobacillus* species composition providing well characterised *Lactobacillus* spp. strains for biotechnological applications.

The molecular methods such as ITS-PCR, controlled by 16S rRNA sequencing are clearly superior to API 50CHL in species identification, particularly for the *Lactobacillus acidophilus* group. For quantitative estimation of *Bifidobacterium* spp. of infants the FISH method has remarkably higher sensitivity in comparison with bacteriological methods. However, for *Lactobacillus* spp. the concordance between bacteriology and FISH methods is high both in infants and adults. In comparison with bacteriological data, higher counts of lactobacilli can be detected in different age groups using FISH and RT-PCR.

2. The composition of intestinal indigenous lactobacilli is characterised with large individual variety both in counts, number and species composition depending on geographical location and age. A geographical comparison of microbiota from Estonian and Swedish 1–2-year-old children showed that the counts of cultivated lactobacilli were significantly higher in the nineties in faecal samples of the Estonians. Considering different age, the faecal microbiota of elderly people in comparison with adults express higher total counts of lactobacilli. In qualitative aspect, the Estonian children are characterised with higher prevalence of particular FHEL group species as *L. plantarum* whereas there is a predominance of *L. paracasei* among Swedish children apparent. In the elderly, the prevalence of the same FHEL species (*L. plantarum* and *L. paracasei*) and the higher species variety can be found whereas in adults the OHOL group (*L. acidophilus* and *L. helveticus*) is prevalent.

3. The higher counts of intestinal *Lactobacillus* spp. assessed using bacteriological methods can be associated with higher BMI and blood glucose content in adults and the elderly. A higher BMI in both groups of persons is predicted by the presence of OHOL species and *L. sakei.* Moreover, lower blood glucose levels are associated with *L. paracasei* in adults and *L. fermentum* in the elderly. Thus, the particular fermentation groups and species of intestinal lactobacilli are specifically associated with human anthropometrical data and biochemical indices within the blood.

4. The previously assessed association between the shifts of prevalence of culturable bifidobacteria in infants and the development of allergy was confirmed with molecular methods in children at 5 years of age. Particularly, in allergic children, *B. adolescentis* is prevalent among intestinal *Bifidobacterium* spp., whereas in non-allergic children *B. catenulatum/pseudocatenulatum* is prevalent. The impact of the different species of *Bifidobacterium* on host metabolism requires further elucidation.

5. Elaborated into probiotics and patented, the *L. plantarum* Inducia DSM 21379 strain was identified using phenotypic (API 50CHL) and genotypic (ITS-PCR, PFGE) methods followed by 16S rRNA sequencing. These methods serve as useful tools for strain identification and typing of lactobacilli from biological material and probiotic products. The concordance between the high proportions of putrescine amounts produced by strain *L. plantarum* Inducia both in decarboxylation media and cheese with an increased metabolism of putrescine is identifiable in its consumer.

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SUMMARY IN ESTONIAN

Soole piimhappebakterite iseloomustus bakterioloogiliste, biokeemiliste ja molekulaarsete meetoditega

Piimhappebakterid (*lactic acid bacteria, LAB*), sealhulgas *Lactobacillus* spp. ja *Bifidobacterium* spp., kuuluvad inimese normaalse mikrobiota hulka. Seedekulgla kasulike mikroobidena omavad nad olulist tähtsust mao- ja sooltrakti mikroobikoosluste tasakaalu säilitamisel. Samal ajal kutsuvad nad oma rakkude ja ainevahetusproduktidega organismis esile üldefekte, mis võivad mõjustada inimese tervist.

Seedekulgla piimhappebakterite liigilist jaotumist ja metaboliite pole erinevatesse eagruppidesse kuuluvatel isikutel, erinevates geograafilistes piirkondades elavatel lasetel ning allergilistel ja mitteallergilistel lastel uuritud. Bakterite metaboolset aktiivsust hinnatakse tavaliselt bakterioloogiliste ja biokeemiliste metoodikate abil. Samas täiendavad molekulaarsed uurimismeetodid arusaamist mikroobide tegelikust mõjust organsüsteemidele. Käesolevas töös iseloomustatakse erineva vanuse, elukoha ja tervisliku seisundiga isikute seedekulglast isoleeritud piimhappebaktereid vastavalt nende feno- ja genotüübilistele omadustele. Pikemaajalise sihina pakuvad saadud tulemused võimalusi uute probiootiliste toodete väljatöötamiseks.

Uurimistöö eesmärgid ja ülesanded

Uurimistöö peamiseks ülesandeks oli iseloomustada bakterioloogiliste, biokeemiliste ja molekulaarsete meetodite abil soolestiku piimhappebakterite liigilist koostist ja metaboolset toimet erineva vanuse, elukoha ja tervisliku seisundiga isikutel.

Uurimistöös püstitati järgmised ülesanded:

1. Võrrelda soole piimahappebakterite kvalitatiivset ja kvantitatiivset koostist bakterioloogiliste, biokeemiliste ja molekulaarsete meetoditega ning nende ainevahetust.
2. Eesti ja Rootsi laste, Eesti tervete täiskasvanute ja eakate isikute seedekulgla *Lactobacillus* spp. liigilise koostise ja hulkade selgitamine.
3. Täiskasvanud ja eakate inimeste seedekulgla *Lactobacillus* spp. hulkade, fermentatsiooni gruppide ja liikide seostamine inimese kehamassi indeksi ja vere glükoosisisaldusega.
4. Seedekulgla *Bifidobacterium* spp. liigilise koostise võrdlus tervetel ja allergilistel 5-aastatel lastel rakendades bifidobakterite genotüpeerimist.
5. Probiootilise tüve *Lactobacillus plantarum* Inducia DSM 21379 genotüübilise samastamismustri, orgaaniliste rasvhapete (SCFA), polüamiinide ja biogeensete amiinide profiili iseloomustamine.

Uuritav materjal ja meetodid

Uurimisalusteks olid 71 Eesti ja 73 Rootsi 1–2 aastast last, 40 5-aastast last (20 allergilist ja 20 mitteallergilist), 24 tervet täiskasvanut ja 37 eakat inimest.

Kokku uuriti 133 laktobatsilli tüve: 35 juhuslikult valitud seedetrakti tüve kultuurikollektsioonist, 76 Eesti ja Rootsi laste seedetraktist isoleeritud tüve, 21 referentstüve ja üht *L. plantarum* Inducia DSM 21379 tüve.

Isoleeritud laktobatsillide tüved samastati liigi tasemel feno- ja genotüübiliste meetoditega (API 50CHL ja ITS-PCR). Laktobatsillide metaboliite määrati gaaskromatograafiaga: orgaanilisi rasvhappeid (äädik-, piim-, ja merevaikhape ja etanool) erinevates keskkonnatingimustes ning polü-ja biogeenseid amiine erinevate aminohapete keskkonnas (arginiin, glutamiin, lüsiin, ornitiin, histidiin), inimese uriinis ja piimatoodetes. Inimeste rooja analüüsides määrati laktobatsillide olemasolu PCR meetodiga ja hulk FISH ning real-time PCR meetoditega.

Allergiliste ja mitteallergiliste laste bifidobakterite liikide määramiseks kasutati liigi-spetsiifilist PCR-DGGE meetodit, järgnevalt kloneerides ja sekveneerides PCR üksikuid bände.

L. plantarum Inducia DSM 21379 samastati liigi tasemel fenotüübilise laktobatsillide API 50CHL samastamiskitiga ning molekulaarselt ITS-PCR-ga, võrdluses tüüptüvedega ja uuritavate tüvede 16S rRNA sekveneerimisega. Laktobatsilli Inducia tüve molekulaarsed näpujäljed määrati PFGE meetodi abil.

Täiskasvanute ja eakate kehamassi indeks arvutati kehakaalu ja pikkuse ruudu suhte alusel. Vereplasma glükoosisisaldus (mmol/L) määrati vastavalt standarditele Tartu Ülikooli Kliinikumi ühendlaboris.

Uurimistöö kokkuvõtte

Erineva vanuse, geograafilise päritolu ja allergilise staatusega isikute seedekulgla piimhappebakterite liigilise koostise ja metaboolse aktiivsuse määramiseks kasutati bakterioloogilisi, biokeemilisi ja molekulaarseid meetodeid.

1. Erinevates biotehnoloogilistes rakendustes kasutatavate laktobatsillide omaduste kirjeldamiseks sobivad fenotüübilised meetodid, mis põhinevad bakterite ainevahetusel, nagu biokeemilise profiili kindlakstegemine API 50CHL-ga ja metaboliitide määramine gaaskromatograafiaga.

Laktobatsillide liikide, eelkõige *Lactobacillus acidophilus* gruppi kuuluvate bakterite määramiseks on molekulaarsed meetodid nagu ITS-PCR ja 16S rRNA sekveneerimine võrreldes API 50CHLga selgelt eelistatavamad. *Bifidobacterium* spp. hulga määramiseks imikutel on FISH meetod märkimisväärselt kõrgema tundlikkusega kui bakterioloogiline meetod. Kuid *Lactobacillus* spp. hulga määramisel nii lastel kui täiskasvanutel ühtib bakterioloogilise ja FISH meetodi kõrge tundlikkus. Erinevates vanuserühmades on laktobatsillide hulgad

FISH ja real-time PCR meetoditega määramisel kõrgemad võrreldes bakterioloogiliste meetoditega.

2. Erinevate inimeste soole indigeensete laktobatsillide hulkade ja liikide mitmekesisus on seotud erinevate geograafiliste piirkondadega ja inimese vanusega. Eesti ja Rootsi 1–2-aastaste laste mikrofloora võrdlusel oli 1990-ndate aastate alguses Eesti laste roojaanalüüsidest isoleeritud laktobatsillide hulk märgatavalt kõrgem kui rootslastel. Eesti eakatel isikutel oli laktobatsillide üldhulk kõrgem kui täiskasvanutel. Leiti erinevusi fakultatiivsete heterofermentatiivsete laktobatsillide (FHEL) levimuses, näiteks sagedasem *L. plantarum* (Eesti lastel) või *L. paracasei* (Rootsi lastel) leid. Eakatel inimestel esines võrreldes täiskasvanutega rohkem ja suuremates hulkades erinevaid laktobatsillide liike, sagedamini leiti samuti FHEL grupi *L. plantarum* ja *L. paracasei* liike. Samas domineeris täiskasvanutel OHOL grupp (*L. acidophilus, L. helveticus*).

3. Bakterioloogiliseltl määratud *Lactobacillus* spp. kõrgemad hulgad seedekulglas seostuvad täiskasvanutel ja eakatel isikutel kõrgema kehamassi indeksi ja vere glükoosi sisaldusega. Mõlema vanusegrupi kõrgem kehamassiindeks on prognoositav OHOL grupi laktobatsillide ja *L. sakei* esinemise põhjal; madalamat veresuhkru taset ennustab täiskasvanutel *L. paracasei* ja eakatel *L. fermentum* esinemine. Seega võib järeldada, et teatud kindlatesse fermentatsioonigruppidesse ja liikidesse kuuluvate laktobatsillide esinemine seedekulglas seostub inimese antropomeetriliste andmete ja vere biokeemiliste näitajatega.

4. Molekulaarsete metoodikatega kinnitusid eelnevate bakterioloogiliste uuringute tulemused allergilistel ja mitteallergilistel 5 aastastel lastel. Allergilistel lastel prevaleerib seedekulgla bifidobakterite perekonnas *B. adolescentis*, samas kui mitte-allergilistel lastel on sagedasem *B. catenulatum/pseudocatenulatum*. *Bifidobacterium* spp. erinevate liikide mõju peremeesorganismi ainevahetusele vajab edasist selgitamist.

5. Probiootikumina patenteeritud tüvi *Lactobacillus plantarum* Inducia DSM 21379 samastati fenotüpeerimis- (API 50CHL) ja genotüpeerimismeetoditega (ITS-PCR, PFGE), millele lisandus 16S rRNA sekveneerimine. Need uurimismeetodid on heaks võimaluseks laktobatsillide samastamisel ja tüpiseerimisel bioloogilisest materjalist ja probiootilistest toodetest. Putrestsiini märkimisväärne hulk söötmes ja juustus on vastavuses laktobatsilli tüve Inducia poolse putrestsiini metabolismiga organismis. Seejuures kinnitab atsetüleeritud putrestsiini hulga tõus uriinis putrestsiini suurenenud ringlust probiootilise juustu tarbijail.

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PUBLICATIONS

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Scientific work

The main subject of the research deals with the intestinal microbial qualitative and quantitative studies of healthy and diseased people in various age groups using molecular methods. Determination of lactic acid bacteria and their metabolic products in various biological materials, and dairy products *in vivo* and *in vitro* experiments. The obtained results have been used for characte-

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Publications

Štšepetova J, Sepp E, Kolk H, Lõivukene K, Songisepp E; Mikelsaar M. Diversity and metabolic impact of intestinal *Lactobacillus* sp. in healthy adults and the elderly. *British Journal of Nutrition* 2011; 105: 1235–1244. (Medline)

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Hütt P, Kõll P, **Štšepetova 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(1): 79–90.

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Patentne leiutis: Mikelsaar M, Songisepp E, Smidt I; **Štšepetova J**; Zilmer M; Hütt P; Truusalu K; Kilk K. Isoleeritud mikroorganismi tüvi *L. plantarum* Inducia DSM 21379 kui organismi loomulikku kaitsevõimet tõstev probiootik, seda sisaldav toiduaine ja kompositsioon ning mikroorganismi kasutamine rakulist immuunsust tõstva ravimi valmistamiseks. (2008) Omanik: Tervisliku Piima Biotechnoloogiate Arenduskeskus. (Prioriteedinumber: P200800027)

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Kutseorganisatsioonid

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Teadustöö

Sooletrakti mikroobiökoloogia kvalitatiivsed ja kvantitatiivsed uuringud mitmetes tervete ja haigete inimeste eagruppides, kasutades molekulaarseid metoodikaid. Piimhappebakterite ning nende ainevahetusproduktide määramine erinevates bioloogilistes materjalides ja piimatoodetes *in vivo* ja *in vitro*

katsetes. Saadud tulemusi on kasutatud inimese seedetrakti laktofloora koostise iseloomustamisel ja seostamisel tervise näitajatega. Tulemuste alusel on publitseeritud 14 teadusartiklit ja 32 konverentsiteesi.

Olulisemad publikatsioonid

Štšepetova J, Sepp E, Kolk H, Lõivukene K, Songisepp E; Mikelsaar M. Diversity and metabolic impact of intestinal *Lactobacillus* sp. in healthy adults and the elderly. *British Journal of Nutrition* 2011; 105: 1235–1244. (Medline)

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