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ORAL LACTOFLORA IN CHRONIC PERIODONTITIS AND PERIODONTAL HEALTH

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To my beloved family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals (I–VI). Additional data are also presented.

I Kõll-Klais P, Mändar R, Leibur E, Marcotte E, Hammarström L, Mikelsaar M. Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. Oral Microbiol Immunol 2005; 20: 354–361.

II Kõll-Klais P, Mändar R, Leibur E, Kjaeldgaard M. High levels of salivary lactobacilli in Estonian schoolchildren. Eur J Paediatr Dent 2004; 5: 107–109.

III Kõll-Klais P, Mändar R, Leibur E, Mikelsaar M. Oral microbial ecology in chronic periodontitis and periodontal health. Microb Ecol Health Dis 2005; 17: 146–155.

IV Marcotte H, Kõll-Klais P, Hultberg A, Zhao Y, Gmür R, Mändar R, Mikelsaar M, Hammarström L. Expression of single-chain antibody against RgpA protease of *Porphyromonas gingivalis* in *Lactobacillus*. J Appl Microbiol 2006; 100: 256–263.

V Kõll-Klais P, Mändar R, Mikelsaar M, Leibur E. Suuõõne mikroobi-ökoloogia kroonilise parodontiidi puhul. Eesti Arst 2003; 82: 759–767 (in Estonian).

VI Kõll P, Mändar R, Marcotte H, Leibur E, Mikelsaar M, Hammarström L. Characterization of oral lactobacilli as potential probiotics (manuscript).

ABBREVIATIONS

ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony Forming Unit
CP	Chronic Periodontitis
DGGE	Denaturing Gradient Gel Electrophoresis
DFT	Decayed and Filled Teeth
DMFT	Decayed, Missing and Filled Teeth
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FAO	Food and Agriculture Organization of the United Nations
FHEL	Facultatively Heterofermentative Lactobacilli
GCF	Gingival Crevicular Fluid
GI	Gingival Index
GRAS	Generally Recognized As Safe
LB	Luria-Bertani
MAb	Monoclonal Antibody
MIC	Minimal Inhibitory Concentration
MRS	de Man-Rogosa-Sharpe
MT	Missing Teeth
MUG	4-Methylumbelliferyl-β-D-Galactopyranoside
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical Density
OHEL	Obligately Heterofermentative Lactobacilli
OHOL	Obligately Homofermentative Lactobacilli
PAL	Periodontal Attachment Level
PAL_{all}	Periodontal Attachment Level of All Sites
PAL_{ds}	Periodontal Attachment Level of Diseased Sites
PCR	Polymerase Chain Reaction
PI	Plaque Index
PPD	Periodontal Probing Depth
PPD_{all}	Periodontal Probing Depth of All Sites
PPD_{ds}	Periodontal Probing Depth of Diseased Sites
RACE	Rapid Amplification of cDNA Ends
RgpA	Arginine-Specific Protease of *P. gingivalis* Derived from *rgpA* Gene
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid

RTF	Reduced Transport Fluid
SAI/II	Surface Adhesion Antigen
scFv	Single Chain Variable Fragment
sIgA	Secretory Immunoglobulin A
TGGE	Temperature Gradient Gel Electrophoresis
TSBV	Tryptone Soya Bacitracin Vancomycin
UV	Ultraviolet Light
VH	Variable Region of Heavy Chain
VK	Variable Region of Light Chain
VMGA	Viability Medium, Göteborg, Anaerobically Prepared and Sterilized
WHO	World Health Organization

INTRODUCTION

Chronic periodontitis is a multifactorial disease elicited by a complex of microbial species that interact with host tissues and cells causing the release of a broad array of inflammatory mediators and cytokines, some of which lead to periodontal destruction (Slots, 1977a; Moore *et al*., 1983; Leibur *et al*., 1999; Jin *et al*., 1999; Socransky and Haffajee, 2005). Nearly 700 bacterial taxa, phylotypes and species, can colonize the oral cavity of humans (Paster *et al*., 2001), however it has remained unclear how this multitude of bacteria interfere with each other to maintain health or conversely, to initiate the disease. Several anaerobic gram-negative bacteria and spirochetes have been associated with the etiopathogenesis of chronic periodontitis (Söder *et al.*, 1993; Socransky *et al.*, 1998; Loesche and Grossman, 2001; van Winkelhoff *et al.*, 2002). At the same time these pathogenic bacteria have also been detected in the oral cavity of young children and healthy adults (Tanner *et al*., 2002; van Winkelhoff *et al*., 2002), suggesting different virulence of strains and/or the presence of some host compatible species with the ability to confront pathogens (Griffen *et al*., 1999). Lactic acid bacteria such as *Streptococcus* and *Lactobacillus* species can generate a low environmental pH, an ecological circumstance unsuitable for the growth of many pathogenic bacteria (Doran *et al*., 2004; Sookkhee *et al*., 2001; Annuk *et al*., 2003). Although some species of streptococci (e.g. *Streptococcus sanguis*) have been associated with periodontal health (Hillman *et al*., 1985; Moore and Moore, 1994; Socransky and Haffajee, 2005), the potential beneficial role of lactobacilli has not been thoroughly investigated. Rather, they are considered to have cariogenic potential in oral cavity (Kohler and Bjarnason, 1987; Bowden, 2000).

Lactic acid bacteria are acknowledged as beneficial members of human microbiocenosis in the gastrointestinal and urogenital tract. During last decades they have gained importance as probiotics, live microorganisms that beneficially affect the host by improving its microbial balance (FAO/WHO, 2002). Intensive research has elucidated several mechanisms of the action of probiotics. Despite intensive research in the field of probiotics in gut only limited research is available for oral probiotics. Consumption of milk containing *Lactobacillus rhamnosus* GG and administration of genetically engineered lactobacilli, expressing antibodies against *Streptococcus mutans*, have been shown to reduce caries risk (Näse *et al*., 2001; Krüger *et al*., 2002). It suggests that administration of oral probiotics offers novel possibilities for the prevention and treatment of dental caries, but the use of probiotics has remained obscure in case of chronic periodontitis.

Dental health of Estonian schoolchildren and adults, and pathogenesis of periodontal diseases have been main subjects of research at the Department of Stomatology, University of Tartu (Russak *et al*., 1984; Wolf *et al*., 1996; Leibur

et al., 1997; Leibur *et al*., 1999). The role of lactobacilli in the human microbiota and the probiotic properties of lactobacilli have been investigated at the Department of Microbiology, University of Tartu during many decades (Voronina, 1968; Mikelsaar, 1969; Lencner, 1973; Annuk *et al*., 2003; Mikelsaar *et al*., 2004; Naaber *et al*., 2004; Songisepp, 2005).

The present thesis specifies the potential beneficial role of oral lactoflora in the maintenance of periodontal health, and the development of novel probiotics for periodontal treatment. During the research, associations between microbiological and clinical data were explored, various properties of oral lactoflora were characterized and biologically active single-chain antibodies against *Porphyromonas gingivalis* on the surface of lactobacilli were expressed.

The collection and evaluation of clinical data were performed at the Department of Stomatology, University of Tartu, Estonia, and at clinical dental practice LLC Elva Hambaprotees, Estonia. The microbiological studies were performed at the Department of Microbiology, University of Tartu, Estonia, and the molecular studies were carried out at the Department of Laboratory Medicine, Karolinska Institutet, Sweden.

REVIEW OF LITERATURE

1. Microflora of the healthy human oral cavity

The mouth provides a large number of diverse surfaces, such as soft shedding tissues of the mucosa (lips, cheek, tongue, palate) and hard nonshedding surfaces of the teeth, on which a wide variety of bacterial complexes are able to form. As many as 700 bacterial species are recognized as being present in the human oral cavity (Paster *et al*., 2001; Socransky and Haffajee, 2005). New species are described as more clinical samples are examined and improved techniques for isolation and identification are used.

1.1. Microflora of different habitats

The indigenous microflora, characterized by its quantitative and qualitative composition, is characteristic of distinct habitats (Table 1) and the factors such as the nature of the surface to be colonized, the clinical status of the habitat and host related factors (e.g. genetic background) are important for the subsequent composition of the microflora. Once established, the composition of the indigenous microflora of each site remains relatively stable over time (Marsh, 2003; Mager *et al*., 2003; Socransky and Haffajee, 2005).

Table 1. Intraoral site distribution of some oral microorganisms

Microorganism	Saliva	Tongue	Plaque	
			Supragingival	Subgingival
Gram-positive				
Streptococcus salivarius	+ + +	+ + +	± to +	±
Streptococcus sanguis	+ +	+ +	+ + +	+
Streptococcus mitis	+ +	+ +	+ +	+ +
Streptococcus mutans	+	+	+ to + + +	± to +
Lactobacillus sp.	+	+	+	± to +
Actinomyces sp.	+	+	+ + to + + +	+ +
Peptostreptococcus sp.	± to +	± to +	+	+
Treponema sp.	±	± to +	±	± to +

Table 1. (Continuation)

Microorganism	Saliva	Tongue	Plaque	
			Supragingival	Subgingival
Gram-negative				
Capnocytophaga sp.	+	+	± to +	± to +
Neisseria sp.	+ +	+ +	+	+
Veillonella sp.	+ +	+ +	+ to + +	+ to + +
Fusobacterium sp.	+	+	+	+ to + +
Prevotella melaninogenica	+	+ to + +	± to +	± to +
Porphyromonas gingivalis	± to +	± to +	± to +	± to +
Actinobacillus actinomycetemcomitans	± to +	± to +	± to +	± to +

Symbols: ±, rarely present; +, present in low proportions; ++, present in moderate proportions; +++, present in high proportions
Data adapted from Loesche, 1994 and modified according to Marsh and Martin, 1999; van der Reijden *et al*., 2001; Socransky and Haffajee, 2005.

1.1.1. Salivary microflora

Although saliva contains up to 10^8 colony forming units (CFU) per ml it is not considered to have an indigenous microflora (Marsh and Martin, 1999). The organisms found are derived from the teeth and mucosa by saliva and gingival crevicular fluid (GCF) flow, chewing and oral hygiene, but it has been shown that the composition of the microbiota in saliva is most closely related to that of the dorsum of the tongue (Gibbons *et al*., 1964, Mager *et al*., 2003). *Veillonella parvula*, *Prevotella melaninogenica* and species of *Actinomyces*, *Streptococcus*, *Lactobacillus*, *Fusobacterium*, *Capnocytophaga* and *Neisseria* are frequently found in saliva (Könönen *et al*., 1999; Colloca *et al*., 2000; Smith *et al*., 2001; Mager *et al*., 2003; Socransky and Haffajee, 2005).

1.1.2. Mucosal microflora

The oral mucosa of the lips, cheeks, palate, tongue, the floor of the mouth, and the attached gingiva is colonized by several species of microorganisms. The lips form the border between the skin microflora, which consists predominantly of staphylococci, micrococci and gram-positive rods (e.g. *Corynebacterium* and *Propionibacterium* sp.), and the mouth, which contains streptococci and many anaerobic gram-negative species. Streptococci constitute the highest proportion of the microbiota on the lips, palate and cheeks, with a predominance of *Streptococcus vestibularis*, *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus constellatus* (Marsh and Martin, 1999; Mager *et al*., 2003). The

genera of *Actinomyces* (that is major oral coryneform), *Neisseria*, *Haemophilus*, *Capnocytophaga, Veillonella*, *Eubacterium*, *Lactobacillus*, *Fusobacterium*, *Prevotella* and *Candida* have also been isolated. Desquamation ensures that the bacterial load on most mucosal surfaces is light. The concentration of micro-organisms on the cheek and palate epithelium is estimated to be 5–25 bacteria per epithelial cell. On the dorsum of the tongue, with its highly papillated surface, higher bacterial density (100 bacteria per epithelial cell) and diversity has been described (Marcotte and Lavoie, 1998; Marsh and Martin, 1999). As for the other mucosal surfaces, streptococci are the predominant bacteria, with *Streptococcus salivarius* predominating (Kazor *et al*., 2003). In addition, high proportions of *V. parvula*, *P. melninogenica*, *Eikenella corrodens*, *Neisseria mucosa*, *Actinomyces odontolyticus*, *Fusobacterium periodonticum*, *Fusobacterium nucleatum* ssp. *vincentii* and *P. gingivalis* have been described on lateral and dorsal surfaces of the tongue in healthy adults (Mager *et al*., 2003). Other microorganisms isolated from the tongue include species of *Peptostreptococcus*, *Eubacterium*, *Lactobacillus*, *Haemophilus*, *Capnocytophaga*, *Gemella*, *Rothia*, *Selenomonas*, *Campylobacter*, *Bacteroides* and *Treponema* (Ahrné *et al*., 1998; Tanner *et al*., 2002; Mager *et al*., 2003; Kazor *et al*., 2003). Studies suggest that soft tissues, particularly the tongue, may serve as reservoirs for infection or reinfection of the periodontium and therefore deserve therapeutic attention (Quirynen *et al*., 2001).

1.1.3. Tooth associated microflora

Dental plaque is a general term for a complex of microbial community found on the tooth surface, embedded in a matrix of polysaccharides, proteins, nucleic acids and phospholipids, and water. Dental plaque develops preferentially on surfaces protected from mechanical friction, such as the area between two teeth (approximal surface), the pits and fissures of the occlusal surfaces, and the subgingival area (gingival crevice). Dental plaque formation involves an ordered colonization (microbial succession) by a range of bacteria. Immediately following professional tooth cleaning, a thin host-derived layer, called the acquired pellicle, covers the tooth surface and is a source of receptors recognized by the primary colonizers of dental plaque. These receptors include mucins, agglutinins, proline-rich proteins, phosphate-rich proteins, such as statherin, and enzymes, such as alpha-amylase. Streptococci, particularly the *S. mitis* group (e.g. *S. oralis*, *S. mitis*, *Streptococcus gordonii*, *Streptococcus sanguis*) and *Actinomyces* sp. are able to bind to these receptors and are considered as the primary colonizers. These primary colonizers co-aggregate with other genera such as *Capnocytophaga* sp., *Haemophilus* sp., *Eikenella* sp., *Prevotella* sp., *Propionibacterium* sp. and *Veillonella* sp. and form a group of early colonizers. Consequently, these early colonizers are thought to prepare the environment for later colonizers (e.g. spirohetes, such as *Treponema denticola*, and various

anaerobic gram negative rods) that have more fastidious requirements for growth. It has been proposed that *F. nucleatum* acts as a bridge between early and late colonizers (Kolenbrander *et al*., 2002).

In general, there are two different types of plaque. Supragingival plaque forms above the gingival margin and subgingival plaque forms below this point.

1.1.3.1. Supragingival microflora

In pits and fissures of the occlusal surfaces of teeth, the microflora is mainly gram-positive and is dominated by streptococci, particularly *S. sanguis* and mutans streptococci. Species of *Actinomyces*, *Staphylococcus*, *Propionibacterium*, *Lactobacillus*, *Eubacterium* and *Veillonella* are also present, but in lower proportions than streptococci. *Neisseria* sp. and *Haemophilus* sp. have been isolated on some occasions. The number of microorganisms colonizing fissures varies, but up to 10^6 CFU per fissure have been found (Marsh and Martin, 1999).

In plaque of the approximal tooth surfaces, streptococci and *Actinomyces* sp., such as *Actinomyces naeslundii*, *Actinomyces israelii* and *A. odontolyticus*, form the majority of the microflora (Ximénez-Fyvie *et al*., 2000a). Gram-negative bacteria of the group *Veillonella*, *Neisseria* and *Fusobacterium* are regularly isolated but in lower proportions than streptococci and *Actinomyces* sp. With the maturation of plaque, bacterial diversity and the number of microorganisms increase and more anaerobic microorganism are found (Löe *et al*., 1965).

1.1.3.2. Subgingival microflora

The gingival crevice is a distinct microbial habitat, influenced both by the anatomy of the site and the flow and properties of GCF. The crevice is a narrow groove surrounding the tooth, with a clinical depth of 0.5–3 mm. The bottom of the crevice is made up of the most coronal cells of the junctional epithelium, which are constantly in the process of being sloughed. One lateral wall of the crevice is made up of the tooth structure; the other is the non-keratinized oral sulcular epithelium (Lindhe and Karring, 1993). Once the microorganisms have colonized this subgingival area, they have access to nutrients present in the gingival fluid. In the healthy gingival crevice, the total number of cultivable bacteria ranges from 10^3 to 10^6 CFU/ml. In contrast to the microflora of teeth fissures and approximal surfaces, higher levels of obligately anaerobic bacteria can be found, many of which are gram-negative. Among the genera and species associated with the healthy gingival crevice are the members of *S. mitis*-group and *Actinomyces* sp., such as *A. naeslundii*, *Actinomyces gerencseriae* and *A. odontolyticus* (Ali *et al*., 1997; Ximénez-Fyvie *et al*., 2000a). *Fusobacterium* sp. and *Peptostreptococcus micros* are among the commonest anaerobes found

in the healthy gingival crevice (Dowsett *et al*., 2002). The most commonly isolated black-pigmented anaerobe in the healthy gingival crevice is *P. melaninogenica*, while *Prevotella nigrescens* has also been recovered on some occasions. *P. gingivalis* is rarely isolated from healthy sites (Griffen *et al*., 1998; van Winkelhoff *et al*., 2002).

1.2. Factors controlling the oral microflora

The microbial ecology in the oral cavity is influenced by the combined action of a variety of physicochemical (Table 2), host and bacterial related factors (Marcotte and Lavoie, 1998).

Table 2. Key environmental factors affecting the growth of microorganisms in the healthy oral cavity

Factor	Range	Comment
Temperature	35–36ºC	
Oxygen	0–21%	Gradients exist in dental plaque enabling obligate anaerobes to grow.
Redox potential (Eh)	+ 200 to – 200 mV	Gradients exist in dental plaque, lowest value in gingival crevice.
pH	6.75 – 7.25	Plaque pH falls during dietary sugar metabolism; subgingival plaque pH rises during inflammation.
Nutrients	endogenous	Peptides, proteins and glycoproteins in saliva and in gingival crevicular fluid.
	exogenous	Dietary sugars (frequent consumption may facilitate selection of acidogenic and acid-tolerating species).

Data adapted and modified from Marsh, 2000.

The mouth is continuously bathed with saliva and this has a profound influence on the ecology of the mouth. The mean pH of saliva is between 6.75 and 7.25, which favors the growth of many microorganisms. In addition, saliva promotes the growth of bacteria by providing nutrients, removing waste products, and acting as the vehicle for transport of bacterial cells from site to site in the oral cavity. On the other hand, the continuous flow of saliva is also removing a large number of microorganisms from the oral surfaces. Saliva also contains several specific and non-specific defense factors, such as secretory immunoglobulin A (sIgA), lactoferrin, lysozyme, peroxidases, mucins, histatins and cystatins that display a wide spectrum of antimicrobial activity (Schenkels *et al*., 1995; Marcotte and Lavoie, 1998). However, saliva does not usually gain access to the

gingival crevice, and this area of the oral cavity is almost essentially controlled by GCF. The crevicular fluid originates from serum and contains antimicrobial substances including immunoglobulins (IgM, IgG and IgA), complement, and leukocytes. The continuous flow of gingival fluid from the crevice to the oral cavity removes nonadherent bacterial cells, but also provides nutrients for bacterial growth.

In addition, a variety of synergistic and antagonistic bacterial interactions influence the homeostasis of the oral microflora. Coaggregation allows indirect adherence of bacteria on oral surfaces. Utilization of oxygen by facultatively anaerobic bacteria reduces the oxygen concentration and redox potential to levels that allow colonization of anaerobic bacteria. Different bacterial species may also provide nutrients for the each other. For example, the metabolism of carbohydrates by *Streptococcus* and *Actinomyces* species generates lactate, which may be used by *Veillonella* as an energy source. Bacterial interactions, such as competition for adhesion receptors and production of inhibitory substances are among the mechanisms involved in reducing bacterial colonization and preventing bacterial overgrowth. Inhibition of periodontal pathogens by viridans streptococci, due to the production of hydrogen peroxide (Hillman *et al*., 1985), and antimicrobial activity of streptococci, lactobacilli and *Actinomyces* strains due to the production of various organic acids has been reported (Tompkins and Tagg, 1986; Sookkhee *et al*., 2001; Doran *et al*., 2004).

Exogenic factors, such as oral hygiene, diet and antimicrobial factors have also an impact on the microbial ecology. The mechanical removal of plaque by tooth brushing and flossing has been shown to be important in the prevention of dental caries and periodontal diseases (Mathiesen *et al*., 1996; Axelsson *et al*., 2004). Frequent consumption of high sugar diet has been shown to increase the risk of development of dental caries by influencing the composition of dental plaque (Gustafsson *et al*., 1954; Minah *et al*., 1985). Antibiotics that are given for the treatment of different infections may enter the oral cavity via saliva and gingival crevicular fluid and lead to an imbalance in the oral microbiota (Marsh and Martin, 1999). Thus, it is useful to know the antibiotic susceptibility pattern of oral microorganisms, particularly of those which participate in the maintenance of oral health.

1.3. Significance of the oral microflora

The information of the role of the indigenous microflora originates from early studies comparing the physiology of germ-free and conventional laboratory animals, and from the study of humans in whom the microflora has been disrupted by long-term administration of antibiotics. Most studies have focused on the gut microflora, and the role played by the oral indigenous microflora is poorly understood (Marsh, 2000). The composition of the microflora may either protect individuals or render them prone to diseases.

One of the beneficial functions of the indigenous microflora is its ability to provide protection against infections caused by pathogenic bacteria. This property has been termed colonization resistance. The mechanisms involved in colonization resistance include competition for nutrients and attachment sites, production of inhibitory metabolites, and creation of unfavorable environmental conditions for exogenous organisms (Marsh, 2000). Many oral bacteria produce inhibitors, such as hydrogene peroxide, bacteriocins, or change local environmental conditions (e.g. pH), which may exclude exogenous species, and suppress potentially pathogenic bacteria (Hillman *et al*., 1985; Morency *et al*., 2001; Doran *et al*., 2004). Recently, it has been proposed that oral commensal bacteria and mucosal tissues exist in a balanced state due to active signaling between the bacteria and the epithelial cells (Henderson and Wilson, 1998). By this cross-talk the host is able to discern host-associated microbial populations and to prevent the induction of damaging inflammation. It involves several pattern recognition receptors such as the Toll-like receptor family, the lipopolysaccharide binding protein and the cell surface molecule CD14 (Akira *et al*., 2001; Jin and Darveau, 2001).

On the other hand, shifts in the composition of the oral microflora beyond levels compatible with oral health may predispose to diseases. Studies have shown that dental caries is associated with increase in the proportion of acidogenic and aciduric bacteria, especially mutans streptococci (such as *Streptococcus mutans* and *Streptococcus sobrinus*), which demineralize enamel (Bowden, 2000). These bacteria are able to rapidly metabolize dietary sugars to acid, creating locally low pH. Under these conditions the acid-tolerant bacteria become more competitive, whereas most species associated with enamel health are sensitive to acidic environmental conditions. In contrast, the accumulation of plaque around the gingival margin elicits an inflammatory host response (including a rise in pH and increased flow of GCF), which could favor the growth of fastidious obligately anaerobic gram-negative bacteria implicated in periodontal destruction (Marsh, 2003).

2. Changes in the oral microflora in case of chronic periodontitis

2.1. Concept, clinical features and epidemiology of chronic periodontitis

Chronic periodontitis is defined as inflammation of the gingiva extending into the adjacent attachment apparatus. The disease is characterized by loss of clinical attachment due to destruction of the periodontal ligament and loss of adjacent supporting bone (American Academy of Periodontology, 2000; Wiebe and Putnins, 2000).

Clinical features may include combinations of the following signs and symptoms: edema, erythema, gingival bleeding upon probing, suppuration, attachment loss, tooth mobility and tooth loss. Chronic periodontitis may be localized, involving one area of a tooth's attachment, or more generalized involving several teeth or the entire dentition. The severity of the disease is based on the amount of attachment loss and is designated as slight, moderate or advanced (American Academy of Periodontology, 2000).

Chronic periodontitis occurs mostly in adults, but it can be seen in younger people as well. Current epidemiological evidence indicates that severe periodontitis occurs in a few teeth in a relatively small proportion of people in any given age cohort, and the proportion affected increases with age. On the other hand, mild gingival inflammation is common (Russak *et al*., 1984) and many adults have mild to moderate loss of periodontal attachment at some sites of teeth. In Europe, the proportion of 35–44-year-old adults with shallow periodontal pockets (3.5–5.5 mm) ranges from 13% to 57%, and the mean proportion of adults with deep periodontal pockets (>5.5 mm) is 14% (Sheiham and Netuveli, 2002).

2.2. Etiopathogenesis of chronic periodontitis

Chronic periodontitis is a multifactorial disease, but the initiation and progression of periodontitis is thought to be caused by bacteria accumulating in the subgingival plaque. Microbial enzymes, toxins and metabolites can directly harm the host or can provoke a destructive inflammatory response in the gingival tissues due to, for example, the release of lysosomal enzymes during phagocytosis, or to the production of inflammatory mediators and cytokines that can stimulate soft tissues and bone resorption (Boström *et al*., 1998b; Leibur *et al*., 1999; Jin *et al*., 1999). However, the mechanisms that trigger the conversion of gingivitis to periodontitis are not known.

P. gingivalis, a gram-negative anaerobic bacterium, has been identified as a major etiologic agent in the pathogenesis of chronic periodontitis in humans

(Loesche and Grossman, 2001; Lamont and Jenkinson, 2000). Other bacteria such as *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia*, *Tannerella forsythia* and *T. denticola* have also been found in high numbers in diseased patients (Ximénez-Fyvie *et al*., 2000a,b; van Winkelhoff *et al*., 2002; Lõivukene *et al*., 2005). Based on the published data, it is likely that several different periodontal pathogens in combination rather than just one can be found in periodontal lesions (Söder *et al*., 1993; Socransky *et al*., 1998).

A number of virulence factors are expressed by periodontal pathogens. *P. gingivalis*, in particular, is well known for its ability to secrete an abundant array of proteases; one of these, an arginine-specific protease, increases vascular permeability resulting in an increase in the flow of gingival crevicular fluid (Imamura *et al*., 1994) and thereby providing a rich source of nutrients for the subgingival plaque community. Bacterial invasion of gingival epithelial cells has been demonstrated for both *P. gingivalis* (Lamont *et al*., 1995; Sandros *et al*., 1994) and *A. actinomycetemcomitans* (Meyer *et al*., 1991; Sreenivasan *et al*., 1993). Invasion of cells by *P. gingivalis* has been shown to affect the innate host inflammatory response to bacteria (Darveau *et al*., 1998).

In the healthy gingival crevice, periodontal pathogens such as *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* are undetectable or found in very low numbers (van Winkelhoff *et al*., 2002; Ximénez-Fyvie *et al*., 2000a). According to ecological plaque hypothesis, changes in local environmental conditions in the subgingival region (e.g. increased flow of gingival crevicular fluid, a rise in pH) favor the growth of putative pathogens at the expense of those seen in health (Marsh, 2003). However, the absence of some host compatible species (e.g. those with antimicrobial activity) may be as important in disease initiation or progression as the presence of one or more pathogenic species (Socransky and Haffajee, 2005). Hence, from the therapeutic point of view, it is essential to clearly define these potential beneficial bacteria in order to control periodontal pathogens without deleterious changes in the remaining ecosystem.

2.3. Shifts in oral microflora composition in chronic periodontitis patients

Evidence that an increase in plaque mass around the gingival margin elicits an inflammatory host response was derived from an experimental gingivitis model (Löe *et al*., 1965). Cessation of oral hygiene measures in periodontally healthy subjects caused accumulation of plaque and development of gingivitis. After recommencement of oral hygiene, gingival inflammation resolved in about a week. When the microflora was studied during the development of gingivitis, young plaque revealed mainly gram-positive cocci and rods. As the plaque matured, gram-negative cocci and rods as well as fusobacteria and spirochetes

appeared and increased in numbers. Subsequent cultural studies have confirmed these results and arrived at the conclusion that gingivitis is associated with an increased microbial load and a corresponding increase in the percentage of gram-negative organisms (Syed and Loesche, 1978; Moore *et al*., 1982a; Moore *et al*., 1984).

Less clear, however, are the factors and pathomechanisms that enable gingivitis to develop to periodontitis. Early cultural studies have shown that with development of periodontitis, there occur both quantitative and qualitative changes in the subgingival microflora. There is an increase in the total microbial load (10^5–10^8 CFU/ml) and a shift from a scanty microflora, dominated by gram-positive organisms and facultatively anaerobic species seen in health, to increased numbers of gram-negative rods and anaerobic species in periodontitis (Slots, 1977a,b; Moore *et al*., 1983; Moore and Moore, 1994; Darveau *et al*., 1997). Several different microbial complexes have been described in the subgingival microflora that are associated with disease. These complexes include those of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *T. denticola*; also *F. nucleatum*, *T. forsythia* and *Campylobacter rectus*; and *P. gingivalis*, *T. forsythia* and *T. denticola* (Socransky *et al*., 1988; Söder *et al*., 1993; Socransky *et al*., 1998).

Although several studies address putative periodontal pathogens, less is known about the microorganisms that might participate in the maintenance of periodontal health. Extensive culture studies by Moore *et al*. (1983, 1994) revealed that the proportion of certain gram-positive and gram-negative species decreased with increasing severity of the disease (Table 3). In particular, various species of *Streptococcus* and *Actinomyces* have been associated with gingival health (Tanner *et al*., 1996; Ali *et al*., 1997; Darveau *et al*., 1997; Ximénez-Fyvie *et al*., 2000a). In another study, Hillman *et al*. (1985) showed that the presence of the periodontal pathogens *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* in the subgingival plaque of periodontitis patients was correlated with the absence of certain streptococci (e.g. *S. sanguis*). Investigating mutans streptococci in the subgingival plaque of periodontitis patients in different stages of periodontal therapy, van der Reijden *et al*. (2001) found an increase in their proportions following therapy, as well as a negative correlation between mutans streptococci and *P. gingivalis*. A recent study by Doran *et al*. (2004) revealed the ability of streptococci to inhibit the growth of various anaerobic bacteria, including the periodontal pathogens *P. intermedia* and *P. gingivalis*. In addition to streptococci, scarce data indicate that also other lactic acid producing bacteria, such as lactobacilli, exert antibacterial activity against *P. gingivalis* (Sookkhee *et al*., 2001). Thus, we may suppose that colonization of the oral cavity by lactic acid bacteria may aid in suppression of periodontal pathogens and hence reduce the incidence of chronic periodontitis. However, the existing data do not provide a clear picture of the role of lactic acid bacteria in the maintenance of oral health, particularly as concerns lactobacilli.

Table 3. Bacteria whose proportions in plaque decrease with chronic periodontitis

Gram-positive bacteria	Gram-negative bacteria
Streptococcus sanguis	*Veillonella parvula*
Streptococcus oralis	*Gemella morbillorum*
Streptococcus intermedius	*Capnocytophaga gingivalis*
Streptococcus gordonii	*Haemophilus segnis*
Actinomyces naeslundii	*Haemophilus aphrophilus*
Actinomyces odontolyticus	*Neisseria mucosa*
Actinomyces meyeri	*Neisseria elongata*
Actinomyces sp.	*Leptotrichia* sp.
Rothia dentocariosa	
Eubacterium saburreum	

Data adapted and modified from Moore and Moore, 1994, and Marsh and Martin, 1999.

3. Lactobacilli of the oral cavity

Lactobacilli are gram-positive, rod-shaped, microaerophilic, non-spore-forming, and catalase-negative bacteria with a DNA base composition of less than 53% G + C (guanine plus cytosine) (Kandler and Weiss, 1986). They are members of the lactic acid bacteria, a physiologically diverse group of gram-positive micro-organisms, which produce lactic acid as the major end product during the fermentation of carbohydrates. Lactic acid bacteria comprise around 20 genera, but historically the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* form the core of the group (Axelsson, 2004). The classical way of distinguishing between species of lactobacilli is based on the phenotypic properties of lactobacilli. According to carbohydrate fermentation patterns and growth at certain temperatures, the genus *Lactobacillus* is divided into obligately homofermentative (OHOL), facultatively heterofermentative (FHEL) and obligately heterofermentative (OHEL) subgroups (Kandler and Weiss, 1986; Klein *et al*., 1998). The carbohydrate metabolism of lactobacilli using the commercial kits (like API 50 CHL by bioMérieux) usually allows a taxonomic differentiation between species; however, due to the phenotypic heterogeneity, molecular methods for proper identification of lactobacilli have been suggested. Molecular methods based on 16S ribosomal RNA (rRNA) gene sequence comparison have been shown to be valuable tools for the identification of the lactoflora in the oral cavity (Marchant *et al*., 2001; Byun *et al*., 2004; Chhour *et al*., 2005). In addition, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) have successfully been used for the identification of lactobacilli in the gastrointestinal tract and vagina (Walter *et al*., 2001; Vásquez *et al*., 2002).

3.1. Counts and species composition

Lactobacilli are common inhabitants of the oral cavity and comprise approximately 1% of the cultivable oral microflora (Marsh and Martin, 1999). However, the counts of lactobacilli in saliva vary considerably between different individuals ranging from no detection of lactobacilli to counts as high as 10^6 CFU per ml of saliva (Klock *et al*., 1990; Petti and Tarsitani, 1998; Mäkinen *et al*., 1998; Gábris *et al*., 1999; Motisuki *et al*., 2005). In addition to saliva, lactobacilli are found on the tongue, tooth surfaces and in the gingival crevice (Ahrné *et al*., 1998; Colloca *et al*., 2000). However, little is known of the preferred habitat of lactobacilli in the mouth (Marsh and Martin, 1999). The most common species of *Lactobacillus* are heterofermentative lactobacilli such as *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactbacillus casei* and *Lactobacillus fermentum*, and the homofermentative *Lactobacillus salivarius* (Ahrné *et al*., 1998; Colloca *et al*., 2000; Marchant *et al*., 2001). Several other species that have been isolated from the mouth include *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus oris*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus jensenii*, *Lactobacillus gasseri* and *Lactobacillus agilis* (Ahrné *et al*., 1998; Colloca *et al*., 2000; Smith *et al*., 2001).

At the same time, there occur high geographic variations in the composition of the human microflora, for example, lactobacilli of the intestinal tract are more frequently present and in higher numbers in populations of developing or Eastern European countries rather than in Western countries (Mikelsaar *et al*., 2002). Less is known about geographic differences regarding oral lactobacilli, and, to our knowledge, there are no data for the species composition of the oral lactoflora in Estonians.

3.2. Role in oral diseases

3.2.1. Dental caries

Dental caries is a progressive disease that expands from the initial focus of enamel degradation and the subsequent exposure of the underlying dentine to a point where microorganisms gain access to the tubular network of dentine that extends to the dental pulp. Dissolution of the enamel by organic acids, particularly lactic acid produced by mutans streptococci, is considered to be the primary event in caries development. Lactobacilli are associated with carious dentine and the advancing front of caries lesions rather than with the initiation of the dental caries process (Bowden, 2000). Recent analysis has, however, indicated the complexity of the microbiota, including the lactoflora, associated

with dental caries in children (Becker *et al*., 2002; Munson *et al*., 2004; Chhour *et al*., 2005). Heterofermentative *L. casei*, *L. paracasei*, *L. rhamnosus* and *L. fermentum* have been shown to be the most frequently isolated lactobacilli from caries lesions, followed by other species such as *L. salivarius*, *L. plantarum* and *L. gasseri* (Botha *et al*., 1998; Marchant *et al*., 2001; Buyn *et al*., 2004; Munson *et al*., 2004). At the same time, recent data by Becker *et al*. (2002) suggest that the major secondary pathogens in early childhood caries are not lactobacilli but species of *Bifidobacterium*, which were the most numerous bacteria in their study in both cavitated and deep dentinal caries lesions, outnumbering both *S. mutans* and lactobacilli. Though correlations between the clinical caries scores and lactobacilli in saliva are often significant, lactobacilli counts alone are not considered reliable enough in predicting caries (Kohler and Bjarnason, 1987; Gábris *et al*., 1999; van Palenstein Helderman *et al*., 2001). On the other hand, as lactobacilli belong to the indigenous human microflora, they can be found also in caries-free persons (Toi *et al*., 2000) and their role in supporting human health has been shown repeatedly (Mikelsaar *et al*., 2004).

3.2.2. Chronic periodontitis

The knowledge about lactobacilli in subjects with chronic periodontitis is very scarce. Early cultural studies by Moore *et al*. (1982b, 1983) as well as a study by Moore and Moore (1994) have shown the complexity of the subgingival microflora in adults with chronic periodontitis, including some lactobacilli such as *Lactobacillus minutus*, *Lactobacillus catenaforme*, *L. acidophilus, Lactobacillus uli, Lactobacillus rimae* and some unidentified strains. Of these, *L. minutus, Lactobacillus* D-2, *L. uli* and *L. rimae* were isolated more frequently and in higher proportions from subgingival samples in subjects with chronic periodontitis than in adults with the healthy periodontium. The authors suggested that these lactobacilli may be associated with the development of periodontitis. However, based on comparative sequence analyses of the 16S ribosomal RNA gene, *L. minutus* and *L. rimae* have now been included in the genus *Atopobium*, and *L. uli* has been reclassified as *Olsenella uli* (Collins and Wallbanks, 1992; Dewhirst *et al*., 2001). Recently, the presence of a few isolates of *L. casei*, *L. brevis* and *L. catenaforme* in the subgingival plaque of patients with refractory periodontitis has been described (Paster *et al*., 2001).

Lactobacilli have also been isolated from the saliva of patients with severe periodontitis (Quirynen *et al*., 1999), but no data exist about their species composition. Drake *et al*. (1993) found negative association between the presence of the periodontal pathogen *P. gingivalis* and lactobacilli in the oral cavities of a random sample of blacks over age 65 in North Carolina counties.

In contrast to the above mentioned scanty studies on the relation between the oral lactoflora and periodontitis, lactobacilli have been shown to play an important role in the maintenance of health in the other parts of the human body

by stimulating the natural immunity as well as by contributing to the balance of microflora (Perdigon *et al*., 2001; Mikelsaar *et al*., 2004). For example, the microbial imbalance between lactobacilli and gram-negative anaerobes in the vagina has been shown to result in the syndrome of bacterial vaginosis (Spiegel, 1991), although its underlying mechanisms are not entirely understood. A study by Naaber *et al*. (1997) showed the protective role of lactobacilli against *Clostridium difficile* infection in gut.

Thus, based on the published data, the role of lactobacilli in chronic periodontitis is unclear. A more profound knowledge about oral lactobacilli could help understand the ecological imbalance in periodontitis and might provide future measures for successful control of the disease.

3.3. Antimicrobial activity

Antimicrobial activity of the members of the indigenous microflora is believed to be an important mechanism of protection against infections. The ability of lactobacilli to inhibit the growth of various infectious agents in gut has been shown, although the mechanisms are not entirely understood (Annuk *et al*., 2003; Jacobsen *et al*., 1999). Lactobacilli produce several antimicrobial compounds, including organic acids (lactic acid, acetic acid, succinic acid), hydrogen peroxide and bacteriocins (Ouwehand and Vesterlund, 2004).

Little is known about the antimicrobial activity of oral lactobacilli and the available data shows contradictory results. Sookkhee *et al*. (2001) analysed the antimicrobial activity of lactic acid bacteria isolated from the healthy oral cavity of Thai volunteers and found that five salivary *Lactobacillus* isolates (3 strains of *L. paracasei* and 2 of *L. rhamnosus*) could inhibit a number of oral bacteria, including the periodontal pathogen *P. gingivalis*. In another study, the ability of oral lactobacilli to inhibit the growth of enteropathogenic bacteria was shown (Smith *et al*., 2001). On the contrary, Testa *et al*. (2003) found no antagonistic interactions between oral lactobacilli (*L. casei*, *L. rhamnosus*, *L. plantarum* and *L. salivarius*) and the anaerobes *P. intermedia* and *F. nucleatum*. Comparing the antimicrobial activity of lactobacilli isolated from subjects with or without carious cavities Ahumada *et al*. (2003) found that lactobacilli from caries active patients showed higher production of inhibitory substances against streptococci. However, they did not specify which species were investigated. Thus, the antimicrobial activity of lactobacilli could be related to status of oral health. Yet to our knowledge, there are no data about the antimicrobial activity of oral lactobacilli originating from chronic periodontitis patients.

3.4. Susceptibility to antibiotics

The use of antibiotics to treat periodontitis has been advocated for a number of years. Mechanical periodontal treatment can reduce total supra- and subgingival bacterial mass, but major pathogens may escape from treatment due to their ability to invade periodontal tissues (Meyer *et al*., 1991; Lamont *et al*., 1995) or because they reside at sites inaccessible to periodontal instruments. In these cases, antibiotics, delivered either locally or systemically, are used as a valuable adjunct to mechanical therapy by many clinicians. The antibiotics used include inhibitors of cell wall synthesis (e.g. penicillin, amoxicillin), protein synthesis (e.g. tetracycline, doxycycline, clindamycin) and nucleic acid synthesis (e.g. ciprofloxacin, metronidazole) (Goodson, 1994; Pähkla *et al*., 2005). The use of antibiotics may also disturb the indigenous microflora of the body, including lactobacilli in the oral cavity as well as in the gastrointestinal tract and vagina. Hence, it is advantageous to avoid the use of antibiotics that are highly active against lactobacilli. By now, mainly the strains originating from the gut and vagina have been investigated and little information is available concerning the susceptibility of oral lactobacilli to antibiotics. Sookkhee *et al*. (2001) determined the antibiotic susceptibility pattern of 5 lactobacilli strains (3 *L. paracasei* and 2 *L. rhamnosus*) to 29 antibiotics, and found them to be susceptible to a wide variety of antibiotics, including amoxicillin, tetracycline and clindamycin. At the same time, species- and strain-specific variation in the antibiotic susceptibility pattern has been documented for lactobacilli (Mändar *et al*., 2001; Danielsen and Wind, 2003), and therefore, more strains and species of lactobacilli should be used to determine the antibiotic susceptibility pattern of oral lactobacilli.

4. Lactobacilli as a perspective tool for treatment of oral diseases

4.1. Lactobacilli as probiotics of the first generation

Lactobacilli are widely used for the manufacturing of fermented foodstuffs and as such, have been consumed for centuries. During recent decades lactobacilli have gained importance as probiotics. According to the expert panel commissioned by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) probiotics are defined as "*live microorganisms which when administered confer a health benefit on the host*" (FAO / WHO, 2002).

Common probiotics include lactobacilli such as *L. acidophilus*, *Lactobacillus johnsonii*, *L. casei*, *L. delbrueckii* ssp. *bulgaricus*, *L. reuteri*, *L. brevis*,

Lactobacillus cellobiosus, *Lactobacillus curvatus*, *L. fermentum*, *L. plantarum*. In addition, probiotics include other lactic acid bacteria such as (1) gram-positive cocci *Lactococcus lactis* ssp. *cremoris*, *Streptococcus salivarius* ssp. *thermophilus*, *Enterococcus faecium*, *Streptococcus diaacetylactis*, *Streptococcus intermedius*, and (2) bifidobacteria *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium thermophilum* (Gibson, 1999; Songisepp, 2005). Also other microbial species, besides lactic acid bacteria, such as *Bacillus* sp. and yeasts have been used as probiotics (Saarela *et al*., 2000; European Commission, 2001).

Many beneficial effects on health have been attributed to probiotics. These effects range from reducing gastrointestinal disorders to protecting against colon cancer (Isolauri *et al*., 2002), though the underlying mechanisms that cause these effects are still poorly understood. One of the proposed mechanisms of the action of probiotics (e.g. lactobacilli) is their ability to improve colonization resistance to gut pathogens. Metabolic end products (such as organic acids) produced by probiotics render low ecological pH which can interfere with the growth of surrounding microorganisms (Sookkhee *et al*., 2001; Annuk *et al*., 2003). In addition, probiotics compete with pathogens for binding sites and available nutrients, and produce antimicrobial substances (Arihara *et al*., 1996; Saito, 2004). Probiotics can also modulate the immune response and possess antioxidative activity, and they have been shown to reduce allergy (Kalliomäki *et al*., 2001; Perdigon *et al*., 2001; Kirjavainen *et al*., 2003; Kullisaar *et al*., 2003).

Probiotics may be administered as a component of functional food (yoghurt, cheese, milk) or as food additives (e.g. capsules, tablets) consisting of one or more strains.

4.2. Lactobacilli as probiotics of the second generation

Today, there is increasing interest in developing genetically engineered or second generation probiotics, e.g. the application of lactobacilli as vehicles for delivery of both active and passive immunity (Pouwels *et al*., 1998; Seegers, 2002). As an example of active immunity, positive immune response to lactobacilli expressing tetanus toxin fragment C has been achieved in mice (Maassen *et al*., 1999). On the other hand, neutralizing antibodies that are directed towards a pathogen, toxin, cytokine or other agent have proved very valuable and specific tools in medicine. With the emergency of single chain antibody technology, it has become possible to produce neutralizing antibodies from recombinant bacteria, including lactobacilli (Tavladoraki *et al*., 1999; Krüger *et al*., 2002).

Strains of species of *L. casei* and *L. plantarum* have most frequently been used for transformation (Maassen *et al*., 1999; Krüger *et al*., 2002; Seegers, 2002).

4.3. Lactobacilli as probiotics against oral diseases

Despite intensive research conducted in the field of probiotics in the gut, only few studies are available on the effects of probiotics in the mouth (Çaglar *et al*., 2005; Meurman, 2005). Yet preliminary data point to the potential of lactobacilli as probiotics against oral diseases. Consumption of milk products containing lactobacilli has been shown to reduce caries risk and the oral carriage of mutans streptococci (Näse *et al*., 2001; Ahola *et al*., 2002; Nikawa *et al*., 2004). Administration of a second generation probiotic, transformed lactobacilli expressing single-chain antibody fragments (scFv) against *S. mutans*, has been shown to protect rats against the development of dental caries (Krüger *et al*., 2002).

Like in case of caries, little is known about the effect of probiotics on the periodontal microflora and periodontal diseases. Russian scientists have reported a positive effect of *Bifidobacterium* sp. (Grudianov *et al*., 2002) and *L. casei* (Volozhin *et al*., 2004) in the treatment of patients with chronic periodontitis. In Japan, Shibata *et al*. (1998) showed that recombinant single-chain antibodies produced from *Escherichia coli* cells inhibited *P. gingivalis* vesicle-associated hemagglutinating activity. Since hemagglutinin is a major glycoprotein of *P. gingivalis* vesicles and confers the ability to adsorb and penetrate into host tissue cells, the authors concluded that this expression system could provide an abundant source of immunotherapeutic agent for protecting against periodontal diseases. Thus, along the emergence of pathogens multiresistant to antibiotics, probiotics or genetically engineered probiotics may offer novel possibilities for the prevention and treatment of periodontal diseases, which requires further studies.

4.4. Selection of lactobacilli as probiotics

Although lactobacilli are generally recognized as safe (GRAS), several requirements have been proposed for novel probiotic strains. Isolates from healthy humans are advised. Strains should be able to colonize the site in which their beneficial action is expected and survive passage through the acidic gastric environment and tolerate the effects of bile when used in the gastrointestinal tract (Saarela *et al*., 2000). Among other functional properties, the high antimicrobial activity of probiotic strains exerts a supplementary positive effect in eradicating or inhibiting target bacteria. At the same time, significant fermen-

tation type-, species- and strain-specific variability in acid and bile tolerance as well as in antimicrobial activity of lactobacilli has been observed (Jacobsen *et al*., 1999; Annuk *et al*., 2003). Therefore, several strains from various fermentation types and species should be tested to choose the best ones. Furthermore, there is a growing concern about the development of antibiotic resistance in pathogenic microorganisms. The spread of antibiotic-resistant genes among bacterial species may occur through lateral gene transfer (Duncan, 2003; Steidler, 2003) and therefore, it would be useful to know the resistance pattern of the probiotic strains to avoid inducing strains that carry transferable resistance genes.

By now, only scanty research has been performed for selection of probiotic strains for oral health. Comelli *et al*. (2002) examined 23 dairy microorganisms for potential probiotic properties. Two of the tested strains (*S. thermophilus* and *L. lactis*) were able to adhere to saliva-coated hydroxyapatite (the principal chemical component of dental hard tissues) and were successfully incorporated into a biofilm mimicking the dental plaque. Furthermore, the *L. lactis* was able to modulate the growth of oral bacteria. The authors concluded that such properties might prove beneficial in modulating the establishment of cariogenic dental plaque. In another study, Sookkhee *et al*. (2001) investigated 3790 isolates (suspected to be lactic acid bacteria) from 130 volunteers in Thailand and found that five strains of species of *L. paracasei* and *L. rhamnosus* expressed considerable inhibitory effect against other microorganisms, including *P. gingivalis* and oral *Candida*. The authors concluded that these strains could be good candidates for applying them as oral bioprotectants.

However, more *Lactobacillus* species originating from different subjects and countries should be investigated for probiotic properties since there have been shown geographic variations in the composition of the human lactoflora as well as species- and strain-specific differences in probiotic properties of lactobacilli (Mikelsaar *et al*., 2002; Annuk *et al*., 2003; Colloca *et al*., 2000).

AIMS OF THE STUDY

The general aim of the study was to assess the role of the oral lactoflora in the maintenance of periodontal health and to develop a potential new intervention mode for chronic periodontitis by using lactobacilli as second generation probiotics.

The specific aims of the study were the following:

1. To compare the qualitative and quantitative composition of the subgingival microflora of chronic periodontitis patients and periodontally healthy subjects in order to determine the differences in lactic acid microorganisms.

2. To determine the presence and counts of subgingival and salivary lactobacilli in chronic periodontitis patients in comparison with periodontally healthy subjects of different age groups.

3. To compare the *Lactobacillus* species composition of the oral cavity of chronic periodontitis patients and periodontally healthy individuals.

4. To determine the antimicrobial activity of the oral lactoflora against periodontal pathogens.

5. To characterize oral lactobacilli *in vitro* as potential probiotic candidates.

6. To develop a second generation probiotic for potential use in the prevention or treatment of chronic periodontitis.

MATERIAL AND METHODS

An overview of the material and methods used in this study is presented in Table 4.

Table 4. Study subjects, microbial strains and performed investigations

Study subjects	Study description	Presented in Papers:
Individuals		
96 schoolchildren	Assessment of salivary lactobacilli counts	II
26 adults with chronic periodontitis	Assessment of oral health, colonization with lactic acid bacteria and subgingival microbial relations	Present study, I, III, V
15 periodontally healthy adults	Assessment of oral health, colonization with lactic acid bacteria and subgingival microbial relations	Present study, I, III, V
Microbial strains		
a) *Lactobacillus* species		
238 oral *Lactobacillus* strains, of these:	Identification by biochemical methods on fermentation type level	I
115 *Lactobacillus* strains	Identification by ARDRA and sequencing of the 16S-rRNA gene; antimicrobial activity assay	I, VI
67 *Lactobacillus* strains	Acid and bile tolerance	VI
22 *Lactobacillus* strains	Antibiotic susceptibility	VI
L. paracasei ATCC 393	Antimicrobial activity, ELISA, FACS and agglutination assays	IV
Transformed *L. paracasei* ATCC 393	Antimicrobial activity, ELISA, FACS and agglutination assays	IV
b) Other microorganisms		
S. mutans NG8	Antimicrobial activity assay	I, III, VI
A. actinomycetemcomitans 31-1-1A	Antimicrobial activity assay	III
A. actinomycetemcomitans 31-2-1A	Antimicrobial activity assay	Present study, I, III, VI

Table 4. (Continuation)

Study subjects	Study description	Presented in Papers:
P. gingivalis ATCC 49417	Antimicrobial activity assay	Present study, I, III, VI
P. gingivalis W83	Antimicrobial activity assay	III, IV
P. intermedia ATCC 25611	Antimicrobial activity assay	Present study, I, III, VI
C. albicans 048	Antimicrobial activity assay	VI

5. Subjects

5.1. Schoolchildren

Ninety-six schoolchildren of grade 6 (54 girls and 42 boys, mean age 12.2 ± 0.5 years) were included in the study (Paper II). They attended the Mart Reinik Gymnasium in Tartu. The oral hygine and the dietary data of the schoolchildren were recorded using questionnaires and are presented in Table 5.

Table 5. Dental hygiene and dietary data of study subjects

	Children (n=96)		Adults with CP (n=26)		Healthy adults (n=15)	
	n	%	n	%	n	%
Dental hygiene habits						
Toothbrushing frequency						
some times a week	4	4	1	4	0	0
once a day	37	39	5	19	2	13
twice or more than twice a day	55	57	20	77	13	87
Use of additional devices for toothbrushing (toothpicks, dental floss)*						
no	87	91	21	81	6	40
yes	9	9	5	19	9	60
Dietary habits						
Meal per day						
twice a day	9	9	4	15	3	20
3 times a day	70	73	16	62	6	40
>3 times a day	17	18	6	23	6	40
Sweets consumption						
3–4 times a week or less	44	46	11	42	4	27
1–3 times a day	41	43	10	39	9	60
>3 times a day	11	11	5	19	2	13

Difference in usage of additional devices: chronic periodontitis (CP) patients vs. periodontally healthy adults *$P<0.05$

5.2. Adults with chronic periodontitis

The study group comprised 26 chronic periodontitis patients (16 female, 10 male, mean age 47.2 ± 11.3 years), with no history of systemic disease or antibiotic therapy within the 6 months prior to sampling (Papers I, III, V). Chronic periodontitis patients were diagnosed as having chronic periodontitis based on gingival inflammation, periodontal breakdown with pocket depth ≥5 mm and radiographic evidence of bone loss. They were consecutively drawn from the waiting list of patients who were referred to the Department of Oral Surgery at the Clinic of Stomatology of Tartu University for diagnosis and treatment of periodontitis. All patients were screened for their suitability and selection of sampling sites a day prior to collection of microbiological samples. Nine periodontitis patients were non-smokers, 2 were current smokers and 15 were former smokers who had stopped smoking on average 16.1 (± 7.5) years ago. The oral hygiene and the dietary data of the periodontitis patients were recorded using questionnaires and are presented in Table 5. None of the subjects were regular users of probiotics.

5.3. Periodontally healthy adults

The study group comprised 15 periodontally healthy subjects (7 female, 8 male, mean age 37.5 ± 10.4 years), with no history of systemic disease or antibiotic therapy within the last six months prior to sampling (Papers I, III, V). Periodontally healthy individuals were defined as having no radiographic or clinical evidence of attachment loss. They were drawn from the list of subjects who came to LCC Elva Hambaprotees for dental check-up. Among the healthy subjects 13 were non-smokers and 2 were former smokers who had stopped smoking on average 3.0 (± 1.4) years ago. The oral hygine and the dietary data of the healthy subjects were recorded using questionnaires and are presented in Table 5. None of the subjects were regular users of probiotics.

Informed consent was obtained from all subjects, in accordance with the procedures of the Ethics Review Committee on Human Research of the University of Tartu.

6. Microbial strains tested *in vitro*

6.1. Lactobacilli strains

The oral *Lactobacillus* isolates used in the present investigations were isolated from the salivary and subgingival samples of chronic periodontitis and periodontally healthy subjectes described above. In total, 238 oral lactobacilli strains were identified on fermentation type level. Of these, 115 (48 strains from chronic periodontitis patients and 67 from healthy adults) were selected for further studies, identified on species level and tested *in vitro* for antimicrobial activity, acid and bile tolerance, and antibiotic susceptibility (Table 4; Papers I, VI).

In addition, the American Type Culture Collection (ATCC) strain *L. paracasei* 393 and transformed *L. paracasei* 393 were used for enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), agglutination assays and antimicrobial activity testing (Paper IV).

6.2. Other microbial strains

S. mutans NG8, *A. actinomycetemcomitans* 31-1-1A, *A. actinomycetemcomitans* 31-2-1A, *P. gingivalis* ATCC 49417, *P. gingivalis* W83, *P. intermedia* ATCC 25611 and *Candida albicans* 048 were used in the present study (Table 4). Of these, *A. actinomycetemcomitans* strains were isolated from a chronic periodontitis patient described above, *S. mutans* NG8, *C. albicans* 048 and *P. gingivalis* W83 belonged to the culture collection strains of the Department of Laboratory Medicine (Karolinska Institutet), and *P. gingivalis* ATCC 49417 and *P. intermedia* ATCC 25611 were kindly provided by Prof. Charlotta Edlund (Division of Clinical Bacteriology, Karolinska Institutet).

7. Clinical examination of the study subjects

7.1. Schoolchildren

Dental caries was diagnosed using the criteria of WHO (WHO, 1997). In addition, incipient caries as “white spot” lesion on smooth surface and dental plaque (Silness and Löe, 1964) were registered (Paper II).

7.2. Adults

The baseline examination included the registration of dental plaque, gingival inflammation and presence of suppuration on probing at four sites (distal, mesial, lingual and buccal sites), and periodontal probing depth and periodontal attachment level at six sites (distal, mid and mesial aspects for both buccal and lingual sites) of each tooth, excluding third molars (Papers I, III, V).

The plaque status of an individual was given as plaque index (PI) according to Silness and Löe (1964) and as the frequency of plaque positive surfaces expressed as a percentage of the total number of surfaces. Gingival inflammation was given as modified gingival index (GI: 0, healthy gingiva with no bleeding on probing; 1, "pin prick" bleeding on probing; 2, immediate and overt bleeding on probing; 3, spontaneous bleeding) (Löe and Silness, 1963; van Winkelhoff *et al*., 2002) and as the frequency of bleeding sites on probing, expressed as a percentage of all sites. Suppuration on probing was recorded as present or absent and the results were given as the frequency of pus positive sites expressed as a percentage of all sites. Periodontal probing depth (PPD) was measured to the nearest millimeter from the gingival margin to the bottom of the gingival sulcus/pocket and attachment level (PAL) from the cemento-enamel junction to the bottom of the periodontal pocket with a Williams periodontal probe. The mean across all sites formed the PPD and attachment level of the patient, designated as PPD_{all} and PAL_{all}, respectively. Sites with a probing depth of ≥5 mm were defined as diseased sites. The frequency of diseased sites was expressed as a percentage of the total number of sites. The mean PPD and attachment level of diseased sites was designated as PPD_{ds}, and $PAL_{ds,}$ respectively.

Dental caries was diagnosed using the criteria of WHO (WHO, 1997).

The same examiner performed all clinical measurements.

8. Investigation of the subgingival microflora

The subgingival microflora was investigated in 26 chronic periodontitis patients and in 15 periodontally healthy adults (Papers I, III, V).

8.1. Collection and transport of specimens

Subgingival microbial samples were collected from two diseased sites in each chronic periodontitis patient and from two clinically healthy gingival sulci in each periodontally healthy subject by means of a gingival crevice lavage method as described by Boström *et al*. (1998a). Prior to sampling, the area was

isolated with cotton rolls and the supragingival region of the tooth surface to be sampled was cleaned and dried with sterile cotton pellets. Small volumes (10–20 µl) were ejected from a glass ampoule with a cannula into the periodontal pocket about 1 mm from the bottom and aspirated into the ampoule. The total volume of sampling fluid was 250 µl. The ejection and aspiration procedure was repeated four times. Four drops (about 40 µl) were transferred from the ampoule to a vial containing anaerobic transport medium, VMGA III. The samples were processed within 2 h.

8.2. Preparation and cultivation of specimens

After vortex mixing for 30 seconds, samples were tenfold serially diluted in pre-reduced peptone water (Oxoid, Unipath, Basingstoke, UK) and 100 µl of appropriate dilutions were plated onto agar media. The following media were used: Brucella agar (Oxoid), supplemented with horse blood and menadion (Slots, 1986) for anaerobic and facultative anaerobic bacteria; tryptone soya agar (Oxoid), supplemented with yeast extract, horse serum, bacitracin and vancomycin (TSBV) for *A. actinomycetemcomitan*s (Slots, 1982), and de Man-Rogosa-Sharpe (MRS) agar (Oxoid) for lactobacilli and streptococci. Brucella plates were incubated in an anaerobic glove box (Sheldon Manufacturing, Inc., Shel LAB, Cornelius, OR, USA) with a gas mixture of 5% H_2, 5% CO_2, 90% N_2 for 5–6 days. TSBV and MRS plates were incubated in microaerobic conditions (10% CO_2) for 72 h.

8.3. Isolation, identification and counting of microorganisms

Colonies with different morphology were Gram stained and examined microscopically. The microorganisms were identified mostly to the genus level by standard methods (Murray *et al*., 1999), while periodontal pathogens were identified on the species level. *A. actinomycetemcomitans* was differentiated by colony morphology (star-like inner structure) on selective medium, cell morphology and catalase production, inability to ferment sucrose and absence of beta-glycuronidase activity using medium containing MUG supplement (Oxoid). Gram-negative anaerobic rods that formed black-pigmented colonies, were vancomycin resistant, colistin sensitive, indole positive and showed brick red fluorescence under UV light were presumptively identified as *P. intermedia*/ *nigrescens,* and black-pigmented colonies with vancomycin sensitivity, colistin resistance, indole production and no fluorescence as *P. gingivalis*. Gram-positive irregularly shaped non-spore-forming anaerobic or facultatively anaerobic rods were classified respectively as “anaerobic coryneforms” and “aerobic

coryneforms" and included species of *Actinomyces*, *Corynebacterium*, *Propionibacterium* and *Bifidobacterium*.

The total count ($\log_{10}$ CFU/ml – colony forming units per millilitre of crevicular fluid) of microorganisms and the counts of various genera and species were calculated for each patient. The detection level of the various microorganisms was 3 $\log_{10}$ CFU/ml. A proportion (%) of the particular microbe in the total count was estimated (Mikelsaar *et al*., 2004).

9. Investigation of salivary lactobacilli and mutans streptococci

Salivary lactobacilli were investigated in 96 schoolchildren, 20 chronic periodontitis patients and 15 periodontally healthy adults by the Dentocult®LB dip-slide (Orion Diagnostica, Espoo, Finland) method, and mutans streptococci in 14 chronic periodontitis patients and 14 periodontally healthy adults by the Dentocult®SM strip (Orion Diagnostica) method in paraffin-stimulated saliva (Birkhed *et al*., 1981) (Papers I, II, III, V). After 3 days of incubation at 37°C the number of lactobacilli and mutans streptococci per ml saliva was estimated by comparing the slides with a density chart provided by the manufacturer. Dentocult®LB test results were expressed as missing, low ($\leq 10^3$ CFU/ml), medium (10^4 CFU/ml), high (10^5 CFU/ml) and very high ($>10^6$ CFU/ml) counts of lactobacilli. Dentocult®SM test were given as missing, low ($<10^5$ CFU/ml), medium (10^5–10^6 CFU/ml) and high ($>10^6$ CFU/ml) counts of mutans streptococci.

10. Characterization of oral lactobacilli

Molecular identification and characterization of lactobacilli were performed in collaboration with Prof. Lennart Hammarström and Dr. Harold Marcotte, Department of Laboratory Medicine, Karolinska Institutet, Sweden.

10.1. Isolation and provisional identification

Colonies with different morphology (at least 2–3 colonies from each morphological type) were picked from Dentocult®LB dip-slides (salivary samples) and MRS plates (subgingival samples) into MRS broth and incubated in a 10% CO_2 environment for 24–48 h (Papers I, VI). Thereafter, a series of MRS plates were

repeatedly streaked to purify the culture. Provisional identification was based on the ability of the isolate to grow in the MRS broth, gram-positive rod-shaped non-sporing cell morphology and negative catalase reaction (Kandler and Weiss, 1986). A total of 238 isolates were provisionally identified as lactobacilli (112 isolates from 18 chronic periodontitis patients and 126 isolates from 15 periodontally healthy adults).

The 238 strains were further analysed for fermentation type according to their physiological properties: the ability to grow in MRS broth for 24 h in a 10% CO_2 environment at 15°C and 37°C and to produce gas in MRS agar containing 1% glucose. The fermentation of glucose without gas, growth at 37°C and no growth at 15°C identifies obligately homofermentative, growth both at 15°C and 37°C without gas production is characteristic of facultatively heterofermentative, and gas production at 37°C and variable growth at 15°C are characteristic of obligately heterofermentative *Lactobacillus* species. To verify the fermentation type, the isolates were further analysed for sugar fermentation pattern (sorbitol, tagatose, melezitose) and arginine hydrolysis (Kandler and Weiss, 1986).

10.2. Molecular identification

For further studies, 12 chronic periodontitis patients and 11 periodontally healthy adults were randomly selected (Papers I, VI). Based on the data of provisional identification, all strains with different characteristics from each particular individual, in total 115 strains, were subjected to species identification. Of these, 108 strains were isolated from saliva and 7 strains, from subgingival samples.

The species of the lactobacilli were identified using rapid amplified ribosomal DNA restriction analysis (ARDRA) (Ventura *et al*., 2000). Firstly, the strains were grouped according to their phenotypic features (fermentation of 0.5% ribose, resistance to 12 µl/ml vancomycin and production of CO_2 in the presence of glucose), followed by amplification and restriction analysis of the 16S-rRNA gene.

The genomic DNA was obtained by the phenol/chloroform extraction. PCR amplification of the 16S-rRNA gene (1.5 kb) with primers P0 and P6 was carried out using 2.5 U of *Taq* DNA polymerase in a total volume of 25 µl in buffer containing 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl, 0.2 mM (each) deoxynucleoside triphosphates, and 1 µl of template DNA. Three to five PCR reactions were performed for each strain. The PCR product was checked in an 0.8% (v/w) agarose gel and digested with a set of four restriction enzymes (*Sau*3AI, *Hinf*I, *Hinc*II and *Dra*I) provided by Promega (Madison, WI, USA). Digested DNA fragments were separated by electrophoresis in a 2.5% (v/w) agarose gel with 50 bp DNA Ladder (GibcoBRL®,

Gaithersburg, MD, USA) as a molecular weight marker, stained with ethidium bromide and photographed under UV light. The different species were identified by comparison of the restriction patterns with type strains described previously (Ventura *et al*., 2000).

Partial sequencing of the 16S-rDNA fragment was performed for strains with uncertain identity by using ABI PRISM® BigDye™ Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA, USA). The determined 16S-rDNA sequences were compared with the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

10.3. Testing of antimicrobial activity

10.3.1. Antimicrobial activity of lactobacilli against microaerophilic and facultative microorganisms

The antimicrobial activity of lactobacilli (in total 115 strains: 48 from chronic periodontitis patients and 67 from healthy subjects) against the target bacteria *S. mutans* NG8, *A. actinomycetemcomitans* 31-2-1A and *C. albicans* 048 was assessed (Papers I, VI) by the deferred antagonism method (Morency *et al*., 2001). The media used as bottom agar were MRS agar (Merck, Darmstadt, Germany) for *S. mutans* and MRS agar without tri-ammonium-citrate and sodium-acetate (pH 7.1) for *A. actinomycetemcomitans* and *C. albicans* (Annuk *et al*., 2003). The media used as top agar were brain-heart infusion (BHI) agar (Merck) for *S.* mutans, BHI agar enriched with yeast extract (Merck) and haemin (Sigma, Steinheim, Germany) for *A. actinomycetemcomitans* and Sabouraud-2% dextrose (Merck) for *C. albicans*. Lactobacilli were stab-inoculated on the surface of the bottom agar and incubated anaerobically (BBL®*GasPakPlus*™, BBL Microbiology Systems, Cockeysville, MD, USA) for 24 h at 37ºC to develop visible macrocolonies. The target bacteria were precultivated in their appropriate media and suspensions of cells were adjusted to a predetermined optical density (OD 0.10–0.25 at 600 nm depending on the target bacteria used) to yield confluent growth in the top agar. Thereafter, the melted (and cooled to 42°C) top agar was seeded with the precultivated target bacterial suspension and poured over the macrocolonies of lactobacilli. The plates were incubated anaerobically (BBL®*GasPakPlus*™) for *S. mutans* and under microaerobic conditions (BBL®*CampyPakPlus*™, BBL Microbiology Systems, Cockeysville, MD, USA) for *A. actinomycetemcomitans* and *C. albicans* at 37ºC for 24 h to yield inhibition zones.

The tests were performed in duplicate, and the results were reported as the mean width of two inhibition zones measured from the edge of the colony of *Lactobacillus* strain to the margin of the inhibition zone.

10.3.2. Antimicrobial activity of lactobacilli and *S. mutans* against anaerobic bacteria

The antimicrobial activity of lactobacilli (in total 63 strains: 21 from chronic periodontitis patients and 42 from healthy subjects) against the target bacteria *P. gingivalis* ATCC 49417 and *P. intermedia* ATCC 25611 was assessed (Papers I, VI) using a streak line procedure (Annuk *et al*., 2003) on Wilkins-Chalgren blood agar plates (Oxoid). A single line of lactobacilli culture, grown in MRS broth for 48 at 37ºC in a 10% CO_2 environment, was seeded in the middle of the agar plate, and cultivated for 48 h at 37ºC in an anaerobic glove chamber (Sheldon Manufacturing, Inc. Shel LAB) with a gas mixture of $CO_2/H_2/N_2$: 5/5/90%. Target bacteria were cultured in Wilkins-Chalgren broth for 48 h at 37ºC in anaerobic conditions and thereafter, a 10 µl aliquot of target bacterial culture (2.0 on the McFarland turbidity scale) was seeded in triplicate perpendicular to the streak line of lactobacilli. Following incubation of the plates for 72 h at 37ºC in anaerobic conditions, the width of the zone of inhibition (mm) of the target bacteria extending from the culture line of lactobacilli was measured.

In addition, the above described method was also used to determine the antimicrobial activity of *S. mutans* NG8 against target bacteria *P. gingivalis* ATCC 49417 and *P. gingivalis* W83, and *P. intermedia* ATCC 25611; and microaerophilic *A. actinomycetemcomitans* 31-1-1A and 31-2-1A (Paper III).

10.3.3. Antimicrobial activity of periodontal pathogens against lactobacilli

The streak line procedure (Annuk *et al*., 2003) was used to assess the antimicrobial activity of *A. actinomycetemcomitans* 31-2-1A, *P. gingivalis* ATCC 49417 and *P. intermedia* ATCC 25611 against 16 strains of oral lactobacilli (1 strain of *Lactobacillus crispatus*, 2 strains of *L. salivarius*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. fermentum* and *L. oris*, and 3 *L. gasseri*) (present study). The periodontal pathogens were precultivated in their appropriate media and incubation conditions for 48 h at 37ºC, streaked in the middle of the Wilkins-Chalgren blood agar plate and incubated for 48 h at 37ºC in anaerobic (*P. gingivalis*, *P. intermedia*) or microaerobic (*A. actinomycetemcomitans*) conditions. Lactobacilli were cultured on MRS agar for 24 h in microaerobic conditions, suspended in a saline solution (1.0 on the McFarland turbidity scale) and streaked (10 µl) in duplicate perpendicular to the streak line of the test bacteria. Following incubation of the plates for 72 h at 37ºC in anaerobic or microaerobic conditions, depending on the periodontal pathogens used, the width of the zone of inhibition (mm) of lactobacilli extending from the culture line of the periodontal pathogens was measured.

10.4. Testing of acid tolerance

A total of 67 strains isolated from periodontally healthy adults were tested for acid tolerance (Paper VI).

10.4.1. Survival testing

The effect of low pH on the survival of lactobacilli was examined in flat-bottom microwell plates (Costar® 96 Well Cell Culture Cluster, Myriad Industries, San Diego, CA, USA) with MRS broth (Merck) adjusted to pH 3.5 to 1.5 with 6N HCl, and a non-adjusted MRS broth (pH 5.6) as control. Each 180-µl volume of pH adjusted or non-adjusted MRS broth were inoculated with the 20-µl of overnight culture of lactobacilli (containing about 10^6 to 10^7 CFU/ml) and incubated aerobically at 37ºC for 4 h. The number of surviving cells was determined by plating 100-µl of tenfold serially diluted sample onto the MRS agar (Jacobsen *et al*., 1999). In total, 67 strains were tested at pH 3.5 and 3.0, and 31 strains were additionally tested at pH 2.5, 2.0 and 1.5.

10.4.2. Growth testing

In parallel, growth at pH 3.5 to 1.5 was measured as changes in OD in 180-µl volume of pH-adjusted and non-adjusted (as control) MRS broth, inoculated with 20-µl of overnight culture of lactobacilli (containing about 10^6 to 10^7 CFU/ml), at 630 nm (OD_{630}) following 0, 3, 6, 9, 12 and 24 h of incubation at 37ºC in aerobic conditions (Jacobsen *et al*., 1999). In total, 67 strains were tested at pH 3.5 and 3.0, and 23 strains were additionally tested at pH 2.5, 2.0 and 1.5.

10.5. Testing of bile tolerance

The effect of bile salts on the growth of lactobacilli (67 strains from periodontally healthy adults; Paper VI) was examined by adding human bile to MRS broth to a final concentration of 0.08, 0.16, 0.3, 0.6, 1.25, 2.5 and 5.0 % (v/v). A 180-µl volume of bile adjusted and non-adjusted (as control) MRS broth were inoculated with the 20-µl of overnight culture of lactobacilli (containing about 10^6 to 10^7 CFU/ml) and incubated aerobically at 37ºC for 24 h. Changes in OD at 630 nm (OD_{630}) were measured following 0, 3, 6, 9, 12 and 24 h of incubation (Jacobsen *et al*., 1999).

10.6. Testing of antibiotic susceptibility

Twenty two strains isolated from periodontally healthy adults were tested (Paper VI). Minimum inhibitory concentrations (MICs) of inhibitors of cell wall synthesis (amoxicillin, cefoxitin, cefprozil, cefotaxime, vancomycin), protein synthesis (gentamicin, erythromycin, doxycycline, tetracycline, clindamycin, chloramphenicol) and nucleic acid synthesis (ciprofloxacin, metronidazole) were determined by E-test method. Saline solution for suspending bacteria (McFarland 0.5 turbidity standard), Wilkins-Chalgren (Oxoid) agar plates with 5% horse blood and E-test antibiotic strips (AB Biodisk, Solna, Sweden) were used. After 48 h of incubation at 37ºC in an anaerobic glove chamber (Sheldon Manufacturing, Inc. Shel LAB), the elliptical zones of growth inhibition were examined and the MICs were interpreted as the value on the E-test strip scale where the inhibition zone intersected the edge of the strip. The breakpoints (susceptible/resistant) were determined in accordance with National Committee for Clinical Laboratory Standards (NCCLS) guidelines for gram-positive microorganisms (Jorgensen and Turnidge, 2003) as follows: clindamycin and ciprofloxacin (4 µg/ml); amoxicillin and erythromycin (8 µg/ml); gentamicin, doxycycline and tetracycline (16 µg/ml); cefoxitin, cefprozil, vancomycin, chloramphenicol and metronidazole (32 µg/ml); and cefotaxime (64 µg/ml). Strains with MICs equal to or higher than breakpoints were considered as resistant.

11. Development of a second generation probiotic against the periodontal pathogen *P. gingivalis* (Paper IV)

Development of a second generation probiotic was performed in collaboration with Prof. Lennart Hammarström, Dr. Harold Marcotte and Dr. Yaofeng Zhao, Department of Laboratory Medicine, Karolinska Institutet, Sweden.

11.1. Construction of anti-*P. gingivalis* scFv-61BG1.3 expression vectors

Total RNA was extracted from an anti-RgpA monoclonal antibody secreting hybridoma (MAb 61BG 1.3) (Gmür *et al*., 1988). Variable region encoding sequences of both the heavy (VH) and light (VK) chains were amplified using a 5' RACE kit (5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Invitrogen Corporation, Carlsbad, CA, USA). The primers for the 5' RACE of the VH chain were ACRACE1, ACRACE2 and ACRACE3, while the primers mkRACE1, mkRACE2 and mkRACE3 were utilized to amplify the

variable region of the VL chain. The A-tailed resulting PCR product was cloned into a pGEM®-T easy vector with 3'-T overhangs and sequenced. The VH and VK sequences were fused together with a linker gene encoding the amino acid sequence $(G_4S)_3$. Both chains were re-amplified from the cloned 5' RACE products using the primers CLA-RVSC-S, PGVHas-linker, PGVKS-linker and EcoR-PGVKas. The resulting VH and VK PCR products were mixed together and used as a template for a fusion PCR using the primers CLA-RVSC-S and EcoR-PGVKas. The fused PCR products were cloned into a pGEM®-T easy vector after addition of overhang A using *Taq* DNA polymerase. The fused scFv-61BG1.3 encoding sequence was finally cut out from the plasmids using *Eco*RI plus *Cla*I and subcloned into pBluescript II SK (+) (Stratagene, La Jolla, CA, USA) containing an E-tag (pBS-E-tag). For generation of the surface expressed antibody fragments, the scFv-E-tag fragment was amplified using primers antiPgClaS and antiPgEcoAS2 with pBS-E-tag as a template. The PCR amplification product was cut using *Cla*I and *Xho*I and ligated into the *Escherischia coli/Lactobacillus* shuttle vector pLP501-scFv-long anchor, generating pLP501-scFv(61BG1.3)-long anchor. The pLP501 vector contains the constitutive promoter of the lactate dehydrogenase gene and an anchor sequence, encoding the last 244 amino acids of the protease P protein of *L. casei* (Krüger *et al*., 2002). *E. coli* XL10-Gold competent cells (Stratagene) were transformed by heat shock. Transformation of *Lactobacillus* was performed by electroporation. Selection of plasmid positive clones was performed using MRS-plates containing 3 μg/ml erythromycin. At each step of the construction process, the gene expression cassette was sequenced (Applied Biosystems).

11.2. Bacteria and culturing procedures

P. gingivalis W83 was cultivated on Wilkins-Chalgren agar plates and in BHI broth supplemented with yeast extract, haemin and menadione, and incubation was made at 37°C under anaerobic conditions (BBL®*GasPakPlus*™). Non-transformed *L. paracasei* ATCC 393 were cultivated at 37°C on MRS agar under anaerobic condition or in MRS broth under aerobic condition. Lactobacilli containing construct pLP501-scFv(61BG1.3)-long anchor were cultured in MRS agar or broth containing 3 μg/ml of erythromycin. Lactobacilli containing construct pLP501-scFv-long anchor expressing a scFv derived from monoclonal antibody Guy's 13 directed against the SAI/II adhesin of *S. mutan*s was used as a control (Krüger *et al*., 2005).

11.3. Protein extraction

The lactobacilli containing the constructs and non-transformed lactobacilli were cultured as described above to an OD_{600} of 0.8. After washing two times with 10 mM Tris-HCl, pH 8.0, the bacteria were resuspended in 500 µl of 10 mM Tris-HCl, pH 8.0, containing 10 mg/ml lysozyme (Sigma) and incubated at 37°C for 1 h. The samples were disrupted by sonication (6 x 30 s on/off cycles) with a 60% duty cycle and output control put to 5 (Vibracell, Sonics & Materials, Danbury, CT, USA). Debris was removed by centrifugation for 10 min at 10 000 *g*.

11.4. Quantification of scFv produced pLP501-scFv(61BG1.3)-long anchor *Lactobacillus* construct

The amount of scFv in the bacterial extract from the pLP501-scFv(61BG1.3)-long anchor *Lactobacillus* construct was estimated by densitometry using purified E-tag scFv as a standard. The full procedure is described in Paper IV.

11.5. Protein purification of RgpA

Plasmid pJFQ30β, encoding the RgpA adhesin domain (G^{721}-R^{1262}) fused to a $(His)_6$ tag at the N-terminus was a generous gift from Dr. Michael A. Curtis, University of London, United Kingdom (Slaney *et al*., 2002). The recombinant plasmid was expressed in *E. coli* XL10-Gold competent cells (Stratagene). Transformed *E. coli* was grown overnight at 37°C in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin. Cells were harvested by centrifugation and resuspended in 4 ml of 6 mM guanidine-HCl (pH 8.0) and sonicated (3 x 10 s on/off cycles) with a 60% duty cycle and output control put to 5 (Vibracell). The lysate was centrifuged (10 000 *g*, 20 min) and the supernatant was mixed with Talon® metal affinity resins (Clontech Laboratories, Palo Alto, CA, USA), and proteins were subsequently purified according to the user's manual.

11.6. *In vitro* experiments for analyzing the biological activity of the scFv

Different methods were used for analyzing the expression and biological activity of the construct. A detailed description of FACS analysis, Western blot analysis, ELISA, agglutination assays and antimicrobial activity testing is given in Paper IV.

12. Statistical analysis

Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA, USA) and Excel (Microsoft Corporation, Redmond, WA, USA) programs. The following tests were employed: Fisher exact test, *t*-test and Mann-Whitney rank sum test (comparison of different study and bacterial groups), Pearson Product Moment Correlation (correlating the proportion (%) of various subgingival bacteria to various clinical parameters, and the presence of various fermentation groups of lactobacilli to the subgingival presence of periodontal pathogens and to various clinical parameters in adults with or without chronic periodontitis) and Spearman Rank Order Correlation (measuring correlations between the proportions of various microbes in adults with or without chronic periodontitis). The differences were considered significant when P value was < 0.05.

RESULTS AND DISCUSSION

13. Clinical parameters of the study subjects
(Papers I, II, III, V)

The clinical data of the study subjects are shown in Table 6. Seventy-five per cent of the children had experienced dental caries with the mean number of decayed, missing and filled teeth (DMFT) 2.6 ± 2.5. Incipient caries was seen in 65% of the children. These parameters are slightly higher as compared with those reported in the same age group in the Nordic countries where 23% to 49% of 12-year-old children are caries-free and the mean caries prevalence is 1.2 to 2.5 DMFT (Von der Fehr, 1994; Nylander *et al*., 2000). However, as DMFT values for Estonian 12-year-old children were 4.6 in 1991 (Bjerner *et al*., 1992) and 4.1 in 1992 (Marthaler *et al*., 1996) dental caries is showing a declining tendency also in our country.

Table 6. Clinical parameters of the study subjects

	Children (n=96)	Adults with CP (n=26)	Healthy adults (n=15)
	Mean ± SD	Mean ± SD	Mean ± SD
Dental caries			
DMFT	2.6 ± 2.5	15.3 ± 4.8	14.8 ± 5.6
DFT	2.6 ± 2.5	9.2 ± 4.6	12.3 ± 5.9
MT	ND	6.0 ± 4.0**	2.5 ± 2.7**
Plaque			
Sites with plaque (%)	Nd	69.9 ± 20.3***	22.0 ± 14.9***
Plaque index (PI)	1.1 ± 0.3	1.5 ± 0.4***	0.3 ± 0.2***
Inflammation			
Sites with gingival bleeding (%)	Nd	55.0 ± 21.6***	5.7 ± 3.9***
Gingival index (GI)	Nd	1.1 ± 0.3***	0.2 ± 0.1***
Sites with suppuration (%)	Nd	1.3 ± 3.0***	ND***
Diseased sites (%)	Nd	26.1 ± 17.2***	ND***
Periodontal probing depth, mm			
PPD_{ds}	Nd	5.8 ± 0.7***	ND***
PPD_{all}	Nd	3.7 ± 1.0***	1.5 ± 0.2***
Periodontal attachment level[1], mm			
PAL_{ds}	Nd	6.4 ± 1.4	…
PAL_{all}	Nd	4.7 ± 1.6	…

DMFT, decayed, missing and filled teeth; DFT, decayed and filled teeth; MT, missing teeth; PPD_{ds}, periodontal probing depth of diseased sites; PPD_{all}, periodontal probing depth of all sites; PAL_{ds}, periodontal attachment level of diseased sites; PAL_{all}, periodontal attachment level of all sites; ND, not detected; Nd, not determined.

[1]Healthy individuals had no evidence of attachment loss.

Difference in clinical parameters: chronic periodontitis (CP) patients vs. periodontally healthy adults $^{***}P<0.001$, $^{**}P<0.01$.

All adults had experienced dental caries. There were no statistically significant differences in DMFT and the mean number of decayed and filled teeth (DFT) between chronic periodontitis patients and the healthy adults, whereas the mean number of missing teeth was significantly higher in the former group. A marked increase in the amount of dental plaque and gingival inflammation, with higher mean periodontal probing depth was observed in the chronic periodontitis patients as compared with the healthy group ($P<0.001$), which was the expected result reported also by the other investigators (Ximénez-Fyvie *et al*., 2000a; Socransky *et al*., 2002; van Winkelhoff *et al*., 2002).

14. Subgingival microflora in chronic periodontitis and periodontal health (Papers III, V)

14.1. Total counts of subgingival microorganisms

The total bacterial count in chronic periodontitis patients was significantly higher than in periodontally healthy adults (Table 7), confirming the results of previous reports (Zambon, 1996; Ximénez-Fyvie *et al*., 2000a). This could be explained by the different subgingival environmental conditions in health and in disease. As revealed by clinical data, the periodontitis subjects had significantly deeper periodontal sites with obviously larger area for bacterial colonization and the high level of inflammation in diseased sites probably increased the amount of necessary nutrients for bacterial multiplication. Moreover, the possible mechanisms aiming to keep the bacterial numbers under control might have weakened.

14.2. Periodontal pathogens

The acknowledged periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* were only found in chronic periodontitis patients (Table 7). *P. intermedia/nigrescens,* although isolated both from diseased and healthy adults, showed higher prevalence and count in the former group.

Our results are generally in accordance with the earlier data (Zambon, 1996; Socransky *et al*., 1998; van der Reijden *et al*., 2001), however, a higher prevalence of *P. gingivalis* in adults with periodontitis has been reported by some other authors (Söder *et al*., 1993; Boström *et al*., 1998b; Griffen *et al*., 1998; van Winkelhoff *et al*., 2002). This could be explained by differences in the methodology used (culturing vs. molecular techniques), but recently the regional differences in the composition of the subgingival microbiota have been revealed as well (Haffajee *et al*., 2004). Moreover, van Winkelhoff *et al*. (2002) have also shown that in addition to *P. intermedia*, both *A.*

actinomycetemcomitans and *P. gingivalis* may colonize subgingival sites of adults without periodontitis, but with lower prevalence than in adults with periodontitis.

The mean number of periodontal pathogens was significantly higher in chronic periodontitis patients than in healthy subjects (1.50 ± 0.95 vs. 0.47 ± 0.52, P=0.001), since only the periodontitis patients (11/26 vs. 0/15, P<0.01) could harbor more than one periodontal pathogen.

Table 7. Composition of the subgingival microflora in chronic periodontitis (CP) patients and periodontally healthy adults.

	CP patients			Healthy adults		
	Subjects colonized	Count in subgingival sites		Subjects colonized	Count in subgingival sites	
Microorganism	(%)	median	(quartiles)	(%)	median	(quartiles)
Total prevalence/count	100	7.3	(6.6–7.8)***	100	6.3	(5.8–6.5)***
A. actinomycetemcomitans	54**	<3.0	(<3.0–4.2)**	0**	<3.0	(<3.0)**
P. gingivalis	23	<3.0	(<3.0)	0	<3.0	(<3.0)
P. intermedia/nigrescens	73	5.5	(<3.0–6.5)***	47	<3.0	(<3.0–3.8)***
Gram(+) bacteria						
Streptococcus mutans group[1]	64	<3.0	(<3.0–4.8)	83	4.7	(<3.0–5.5)
Streptococcus mitis group[2]	100	5.8	(5.3–6.2)	100	5.5	(4.2–5.8)
Streptococcus anginosus group[3]	73	5.0	(<3.0–5.8)**	67	<3.0	(<3.0–4.3)**
Streptococcus sp.	100	6.1	(5.4–6.5)	100	5.8	(4.8–6.0)
Staphylococcus sp.	31	<3.0	(<3.0)	20	<3.0	(<3.0)
Enterococcus sp.	12	<3.0	(<3.0)	20	<3.0	(<3.0)
Aerobic coryneforms	96	5.8	(4.8–6.5)	100	5.4	(4.7–5.8)
Lactobacillus sp.	0	<3.0	(<3.0)	13	<3.0	(<3.0)
Peptostreptococcus sp.	92	5.6	(4.7–6.8)**	100	4.9	(4.3–5.5)**
Anaerobic coryneforms	92	5.8	(4.6–6.7)***	73	3.9	(<3.0–4.8)***
Eubacterium sp.	62*	<3.0	(<3.0–5.9)	93*	3.9	(<3.0–4.5)
Clostridium sp.	8	<3.0	(<3.0)	7	<3.0	(<3.0)

Table 7. (Continuation)

Microorganism	CP patients: Subjects colonized (%)	CP patients: Count in subgingival sites median (quartiles)	Healthy adults: Subjects colonized (%)	Healthy adults: Count in subgingival sites median (quartiles)
Gram(–) bacteria				
Fam. Neisseriaceae	69	<3.0 (<3.0–4.0)	60	<3.0 (<3.0–4.1)
Fam. Enterobacteriaceae	4	<3.0 (<3.0)	7	<3.0 (<3.0)
Haemophilus sp.	62	<3.0 (<3.0–4.5)	67	<3.0 (<3.0–4.4)
Capnocytophaga sp.	50	<3.0 (<3.0–5.1)	40	<3.0 (<3.0)
Other Gram(–) facultative rods	35	<3.0 (<3.0)	27	<3.0 (<3.0)
Veillonella sp.	81	4.3 (<3.0–5.7)	100	4.7 (4.0–5.3)
Fusobacterium sp.	54	<3.0 (<3.0–5.5)	40	<3.0 (<3.0–3.5)
Campylobacter sp.	42	<3.0 (<3.0–5.4)	47	<3.0 (<3.0)
Other Gram(–) anaerobic rods	100	6.5 (5.8–7.3)***	100	5.0 (4.5–5.7)***
Yeasts *Candida* sp.	4	<3.0 (<3.0)	7	<3.0 (<3.0)

The table shows the prevalence of various species (%) among CP patients and healthy adults, and microbial counts in subgingival sites (log_{10} CFU/ml; detection level 3.0).
Difference in prevalence and microbial counts between the two groups: ***$P \leq 0.001$, **$P < 0.01$, *$P < 0.05$.
[1]*S. mutans, S. sobrinus*
[2]*S. mitis*, *S. oralis*, *S. sanguis*, *S. parasanguis*, *S. gordonii*, *S. crista*
[3]*S. anginosus*, *S. constellatus*, *S. intermedius*

14.3. Composition of the subgingival microflora

Mixed aerobic and anaerobic subgingival microflora was seen in all studied subjects (Table 7). More than 20 different microorganisms were found both in healthy subjects and periodontitis patients, with similar number of species recovered from one subject (mean 10.7 vs. 11.4 in the former and the latter group, respectively).

Despite great individual variations between the subjects, some differences between the groups could be observed. The counts of different gram-negative anaerobic rods, *Streptococcus anginosus* group, peptostreptococci and anaerobic coryneforms were significantly higher in diseased than in healthy individuals. Similar results have been presented in some earlier studies (Slots, 1977a; Moore *et al*., 1983; Socransky *et al*., 1998; Ximénez-Fyvie *et al*., 2000a). In addition, we found that the prevalence of *Eubacterium* species was lower among chronic periodontitis patients as compared with healthy individuals, but with similar counts in both groups. In contrast to our study, increased levels of *Eubacterium* species in diseased periodontal pockets have been observed (Moore *et al*., 1982b; Moore and Moore, 1994; Ximénez-Fyvie *et al*., 2000a). *Eubacterium* species are a group of gram-positive, non-spore-forming, anaerobic bacilli, which are difficult to culture and identify. Downes *et al.* (2001) characterised a total of 105 isolates of *Eubacterium*-like strains and found the majority of the strains to belong to 14 species. The diversity of the organisms belonging to the genus *Eubacterium* could explain the differences between our data and previous observations.

Lactobacilli, although frequently isolated from saliva, were seldom found from subgingival sites – only two out of 15 healthy subjects, but none of the chronic periodontitis patients were colonized, which clearly shows that the subgingival region is not a common habitat for lactobacilli. The counts of subgingival lactobacilli in two *Lactobacillus* positive sites were quite low – 4.5 and 3.5 $\log_{10}$ CFU/ml.

14.4. Relationships between different groups of microorganisms and clinical parameters

In addition to the usual analysis of the prevalence and counts of the species and genera of microbes, the proportions (%) of bacteria in the total count of microorganisms in a particular periodontal site were calculated as suggested formerly for gastrointestinal and vaginal biotopes (Mikelsaar *et al*., 2004). Comparison of the proportions of different microorganisms revealed some significant differences between the study groups and interesting relationships between the microbial groups.

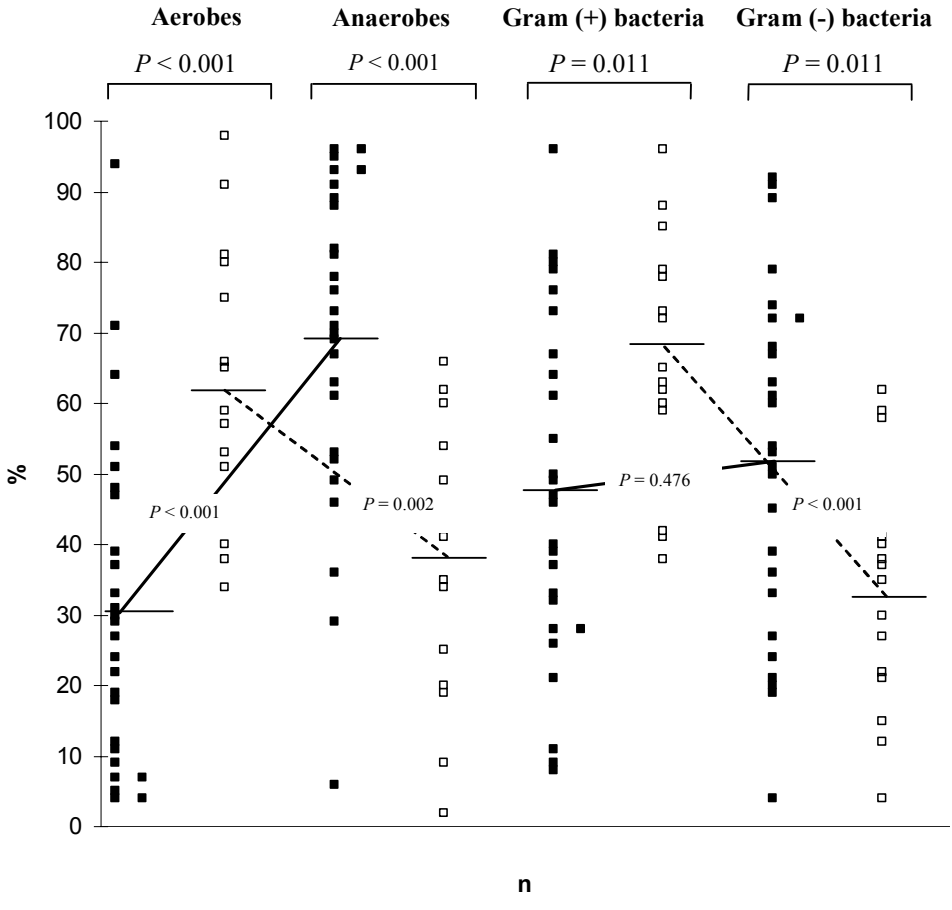

Figure 1. The proportion of aerobic, anaerobic, gram-positive and gram-negative bacteria expressed as a percentage of the total subgingival flora of chronic periodontitis patients (solid squares, n=26) and periodontally healthy subjects (open squares, n=15). Each symbol represents a mean proportion of a particular microbial group in a subject. Perpendicular lines represent a mean value for each group of microorganisms averaged across subjects. All microaerophilic and facultatively anaerobic bacteria were included in the "aerobes" and all obligately anaerobic bacteria in the "anaerobes". Reprinted with permission.

A strong inverse relationship in the proportion of aerobic and anaerobic as well as gram-positive and gram-negative microorganisms was found in periodontally diseased sites of chronic periodontitis patients and in healthy sites of healthy subjects (Fig. 1), indicating significant imbalance in the periodontal microflora of chronic periodontitis patients. These results confirm some earlier findings (Slots 1977a,b; Moore and Moore, 1994). By our data this imbalance has formed at the expense of certain groups of bacteria: the mean proportion of

anaerobic bacteria, particularly gram-negative anaerobic rods (29.9 ± 22.4% vs. 12.7 ± 11.4%, $P<0.001$) and gram-positive anaerobic coryneforms (8.7 ± 10.9% vs. 2.5 ± 3.7%, $P<0.001$), was significantly higher in diseased sites, while the proportion of facultative gram-positive bacteria, particularly streptococci (27.0 ± 23.4% vs. 15.2 ± 19.2%, $P<0.05$) and aerobic coryneforms (24.7 ± 27.5% vs. 11.9 ± 14.7%, $P<0.05$), was significantly higher in healthy sites. Within the group of streptococci, the mean proportions of *S. mutans* group (5.7 ± 9.7% vs. 0.8 ± 1.8%, $P<0.05$) and *S. mitis* group (18.1 ± 18.9% vs. 5.0 ± 5.8%, $P<0.01$) were significantly higher in healthy sites.

There were positive correlations between the proportions of subgingival periodontal pathogens with each other and negative correlations with streptococci and aerobic coryneforms in the subgingival microflora of both chronic periodontitis patients and periodontally healthy adults (Table 8). Similar tendencies were observed when these microorganisms were correlated with chronic periodontitis related clinical parameters.

Proceeding from the above-mentioned data, we checked *in vitro* antimicrobial activity between streptococci and periodontal pathogens. Inhibition of all periodontal pathogens by *S. mutans* was observed, with the strongest antimicrobial activity towards *P. gingivalis* (mean zone of inhibition 22.0 ± 1.4 mm and 17.0 ± 1.0 mm for *P. gingivalis* strains ATCC 49417 and W83, respectively; 12.3 ± 0.6 mm for *P. intermedia* ATCC 25611; and 6.2 ± 0.5 mm and 6.0 ± 0.8 mm for *A. actinomycetemcomitans* strains 31-1-1A and 31-2-1A, respectively). On the other hand, none of the strains of *A. actinomycetemcomitans* and *P. gingivalis* were able to inhibit the growth of *S. mutans*, and only mild antimicrobial activity was expressed by *P. intermedia* (mean zone of inhibition 2.9 ± 0.2 mm). These data are in complete accordance with the above-described relationships between different microorganisms and clinical parameters and suggest that lactic acid producing streptococci as significant antagonists against periodontal pathogens might play an important role in maintenance of a balanced oral microenvironment.

Table 8. Correlations of the proportions of periodontal pathogens and clinical parameters with the proportions of streptococci, aerobic coryneforms and anaerobic gram-negative rods at respective subgingival sites of both chronic periodontitis patients and periodontally healthy adults.

Periodontal pathogens	Oral microbes	Correlation coefficient R	*P*-value
A. actinomycetemcomitans	vs. streptococci	–0.21	NS
	vs. *S. mutans group*	–0.28	0.046
	vs. *S. mitis group*	–0.42	0.002
	vs. aerobic coryneforms	–0.17	NS
	vs. *P. gingivalis*	0.34	0.002
	vs. *P. intermedia / nigrescens*	0.26	0.020
P. gingivalis	vs. streptococci	–0.27	0.014
	vs. *S. mutans group*	–0.28	0.046
	vs. *S. mitis group*	–0.23	NS
	vs. aerobic coryneforms	–0.08	NS
	vs. *P. intermedia / nigrescens*	0.35	0.002
P. intermedia / nigrescens	vs. streptococci	–0.16	NS
	vs. *S. mutans group*	–0.11	NS
	vs. *S. mitis group*	–0.02	NS
	vs. aerobic coryneforms	–0.24	0.034
Periodontal status			
Plaque index (PI)	vs. streptococci	–0.37	0.002
	vs. *S. mutans group*	–0.30	0.032
	vs. *S. mitis group*	–0.33	0.016
	vs. aerobic coryneforms	–0.40	0.001
	vs. anaerobic gram-negative rods	0.45	<0.001
Gingival index (GI)	vs. streptococci	–0.44	<0.001
	vs. *S. mutans group*	–0.31	0.028
	vs. *S. mitis group*	–0.35	0.011
	vs. aerobic coryneforms	–0.30	0.014
	vs. anaerobic gram-negative rods	0.44	<0.001
Periodontal probing depth (PPD)	vs. streptococci	–0.23	0.034
	vs. *S. mutans group*	–0.33	0.016
	vs. *S. mitis group*	–0.39	0.004
	vs. aerobic coryneforms	–0.28	0.012
	vs. anaerobic gram-negative rods	0.36	<0.001

NS, not statistically significant.

15. Salivary levels of lactobacilli and mutans streptococci (Papers I, II, III, V)

15.1. Salivary lactobacilli in schoolchildren

All of the children carried lactobacilli in their saliva. Forty-seven per cent of the children had low, 28% had medium, and 25% had high or very high counts of lactobacilli in saliva. Our data agree with Kohler *et al*. (1987) who reported high counts of lactobacilli in 23% of the 12-year-old Icelandic children. This proportion has been found to be even higher (38%) among Finnish children (Raitio *et al*., 1996). On the other hand, in Sweden, there has been found a decreasing tendency in the number of oral lactobacilli during recent years (Nylander *et al*., 2000): 16% of children were free of lactobacilli in 1998, whereas only 12% of children had high or very high lactobacilli counts. Several studies have shown large geographic variations in the human lactoflora though mainly the gastrointestinal tract was investigated. For example, it has been shown that intestinal lactobacilli are more common in Estonian than in Swedish children, and there is a similar tendency for oral lactobacilli when our data are compared with those of Nylander *et al*. (2000). The Estonian diet is still to a large extent based on locally produced foods and serves, in addition to foodstuffs fermented by lactic acid bacteria, as a possible source of lactobacilli (Mikelsaar *et al*., 2002).

15.2. Salivary lactobacilli and mutans streptococci in adults

All healthy subjects (n=15) and 18 of 20 chronic periodontitis patients harbored salivary lactobacilli. Their counts were significantly higher in adults as compared with schoolchildren ($P<0.05$), but were similar in both adult groups (very high or high count in 60% of healthy and 50% of chronic periodontitis patients, missing or low count in 20% vs. 30%, respectively, and medium count in 20% of both groups).

Mutans streptococci were isolated from all subjects in both groups, however, a trend for an inverse relationship was observed: the counts were low in 64% of chronic periodontitis patients and 36% of healthy subjects, while medium and high counts were seen in 36% of chronic periodontitis patients and 64% of healthy subjects. However, this difference was not statistically significant. In comparison with our data, lower counts of salivary lactobacilli and mutans streptococci have been presented for Swedish adults. Of 718 subjects, 40% and 50% had high lactobacilli ($\geq 10^5$ CFU/ml) and mutans streptococci ($\geq 10^6$ CFU/ml) counts, respectively (Klock *et al*., 1990). In young Estonian adults mean $\log_{10}$ lactobacilli and mutans streptococci scores in saliva (obtained by

Orion diagnostica test kits) have been reported to be 4.2 (± 1.1) and 2.0 (± 0.8), respectively (Mäkinen *et al*., 1998).

16. Characteristics of the oral lactoflora in relation to periodontitis (Papers I, VI, present study)

16.1. Species composition

Altogether 238 *Lactobacillus* isolates (231 salivary and 7 subgingival) from 18 periodontitis patients and from 15 periodontally healthy adults were subjected to further investigations and identified on fermentation type level. The prevalence of different fermentation types of lactobacilli in healthy adults was quite even (OHOL present in 67% of subjects, FHEL in 67% and OHEL in 73%), whereas a twofold decrease of OHOL and a predominance of the FHEL type was seen in chronic periodontitis patients (Table 9).

Further, 115 lactobacilli strains from randomly selected persons were identified by molecular methods, and of those 113 isolates were identified by ARDRA as *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. rhamnosus* and *L. fermentum*. Thirty strains (26%) were later reassigned based on results from the 16S-rRNA gene sequencing (first 500 bases of the16S-rRNA gene). The latter included all strains of *L. casei* to *L. paracasei* ssp. *paracasei* and 14 strains of *L. fermentum* to *L. oris*. Two strains which showed an unknown restriction pattern by ARDRA were identified by sequencing as *L. delbrueckii* and *L. fermentum*.

The distribution of different *Lactobacillus* species among chronic periodontitis patients and healthy subjects is shown in Table 9, with *L. gasseri* and *L. fermentum* being the most prevalent (both 64%) in healthy persons. In comparison with healthy subjects, in chronic periodontitis patients obligately homofermentative lactobacilli, particularly *L. gasseri,* were less prevalent (64% vs. 8% for *L. gasseri, P*<0.01). *L. plantarum*, the most prevalent strain in chronic periodontitis patients, was found in about one-third of healthy subjects. Healthy subjects were colonized by somewhat higher number of species than diseased ones (mean 3.2, range 1–6 species vs. mean 2.1, range 1–4 species), however, this difference was not statistically significant. Similar diversity in the oral lactoflora was observed by Ahrné *et al.* (1998), however they found *L. plantarum* and *L. rhamnosus* as the species most frequently recovered from the tongue mucosa of healthy humans. Colloca *et al*. (2000) found *L. fermentum*, *L. plantarum*, *L. salivarius* and *L. rhamnosus* as predominant species in the healthy human mouth (teeth, tongue, saliva and gum).

Of the seven subgingival *Lactobacillus* strains, originating from two healthy persons, three strains were *L. gasseri*, three were *L. oris* and one was

L. paracasei. The knowledge about subgingival lactobacilli is very scarce. *L. minutus*, *L. rimae* and *L. uli*, isolated from human subgingival sites of subjects with or without periodontitis (Moore *et al*., 1983; Moore and Moore, 1994), have been reclassified as *Atopobium minutum*, *Atopobium rimae* and *Olsenella uli* (Collins and Wallbanks, 1992; Dewhirst *et al*., 2001). Recently, the presence of few isolates of *L. casei*, *L. brevis* and *L. catenaforme* in the subgingival plaque of patients with refractory periodontitis has been described, whereas in contrast to our findings, no lactobacilli were found in 5 periodontally healthy subjects (Paster *et al*., 2001).

Hence, in the present study we showed for the first time that the composition of oral lactoflora between patients with chronic periodontitis and healthy subjects differed, with a lower prevalence of homofermentative lactobacilli, particularly *L. gasseri*, in the former group.

Table 9. Composition of oral lactoflora with respect to periodontal health: prevalence (%) of various fermentation groups and *Lactobacillus* species in chronic periodontitis (CP) patients and periodontally healthy adults harboring lactobacilli.

Lactobacilli		Colonized CP patients		Colonized healthy adults	
Fermentation type / species		n	%	n	%
Identified by biochemical methods		18		15	
OHOL		6	33	10	67
FHEL		16	89	10	67
OHEL		9	50	11	73
Identified additionally by molecular methods		12		11	
OHOL		2*	17	8*	73
	L. acidophilus	0	0	1	9
	L. crispatus	0	0	1	9
	L. delbrueckii	0	0	1	9
	L. gasseri	1**	8	7**	64
	L. salivarius	2	17	3	27
FHEL		11	92	7	64
	L. paracasei	5	42	4	36
	L. plantarum	7	58	4	36
	L. rhamnosus	3	25	2	18
OHEL		5	42	9	82
	L. fermentum	5	42	7	64
	L. oris	2	17	5	45

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative

Difference in prevalence between the two groups: $^{*}P<0.05$, $^{**}P<0.01$

16.2. Antimicrobial activity

The antimicrobial activity between the above-described lactobacilli and putative oral pathogens was investigated as it is known that interactions among microorganisms, both synergistic and antagonistic, influence the homeostasis of the oral microbiota (Marcotte and Lavoie, 1998). We noted a significant antimicrobial activity of lactobacilli. The majority of *Lactobacillus* strains (of both chronic periodontitis patients and periodontally healthy adults) suppressed growth of *A. actinomycetemcomitans* (88% of tested strains), *P. gingivalis* (82%), *P. intermedia* (65%) and *S. mutans* (69%) but none inhibited *C. albicans*. We also investigated the antimicrobial activity inversely. None of the periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* inhibited the growth of the investigated 16 lactobacilli strains of different species.

16.2.1. Fermentation type and species level

The antimicrobial activity was mainly species-specific; however, some strain-specific differences were observed, particularly among strains of *L. fermentum*, *L. oris* and *L. gasseri*. The strongest antimicrobial activity was associated with facultatively heterofermentative lactobacilli and homofermentative *L. salivarius* (Table 10), which is consistent with their phylogenetic relatedness, both belonging to the *L. casei-Pediococcus* group.

In addition, homofermentative *L. crispatus* and a prevalent species in periodontally healthy persons, *L. gasseri*, had quite high activity against anaerobic *P. gingivalis* and *P. intermedia*, whereas another predominant species in healthy persons, heterofermentative *L. fermentum*, inhibited neither of these anaerobic bacteria, but was active against microaerophiles.

Good antimicrobial activity of oral *L. paracasei* and *L. rhamnosus* against *S. mutans* and *P. gingivalis* has previously been shown by Sookkhee *et al*. (2001). In contrast, Testa *et al*. (2003) found no antagonistic interactions between oral lactobacilli (*L. casei*, *L. rhamnosus*, *L. plantarum* and *L. salivarius*) and the anaerobes *P. intermedia* and *F. nucleatum.* As in the present study, no inhibition of *C. albicans* by lactobacilli was seen when Mastromarino *et al*. (2002) characterized vaginal lactobacilli, but some anticandidal effect of strains of *L. paracasei* and *L. rhamnosus* was found by Sookkhee *et al*. (2001). These conflicting results regarding antimicrobial activity could be due to differences in methodology, but could also be related to microbial factors, like the strain-specific antimicrobial activity of lactobacilli as well as variability in sensitivity of different target strains. The antimicrobial activity of lactobacilli could be related to the production of hydrogen peroxide, bacteriocins and organic acids, such as lactic and acetic acid, upon fermentation of glucose with

concomitant decrease in pH (Zhu *et al*., 2000; Mastromarino *et al*., 2002; Annuk *et al*., 2003).

In the present study we also found that lactobacilli from all fermentation types showed higher antimicrobial activity against *P. gingivalis* than against *P. intermedia* (inhibition zone 18.6 ± 5.4 vs. 7.4 ± 3.9 mm, $P<0.001$, in OHOL group; 22.0 ± 3.3 vs. 10.5 ± 1.7 mm, $P<0.001$, in FHEL group; 6.4 ± 7.4 vs. 0 mm, $P<0.05$, in OHEL group), which may partly be caused by the inability of *P. gingivalis* to grow at a pH below 6.5, whereas *P. intermedia* can grow at a pH as low as 5.0 (Takahashi and Schachtele, 1990). Lactic acid, in addition to its antimicrobial effect, resulting from the lowering of pH, also functions as a permeabilizer of the outer membrane of gram-negative bacteria, and so may potentiate the effects of other antimicrobial substances (Alakomi *et al*., 2000).

16.2.2. Origin level

Comparing the lactobacilli of healthy subjects and chronic periodontitis patients, we noted that the antimicrobial activity of heterofermentative lactobacilli was similar (Table 10). At the same time, obligately homofermentative *L. gasseri* strains (n=12, both salivary and subgingival strains) isolated from healthy subjects showed a higher antimicrobial activity against *A. actinomycetemcomitans* (mean 1.6 ± 0.8 mm vs. 0.5 ± 0.5 mm, $P<0.05$) and a lower activity against *S. mutans* (0.1 ± 0.2 mm vs. 1.5 ± 0.7 mm, $P<0.05$) than strains isolated from chronic periodontitis patients.

As a whole, the strains from periodontally healthy subjects showed a lower antimicrobial activity against *S. mutans* than the strains from chronic periodontitis patients (mean 1.2 ± 1.4 mm vs. 2.3 ± 1.4 mm, $P<0.001$). This finding may at least partly explain an inverse association between dental caries and periodontal diseases observed by Sioson *et al.* (2000).

The antimicrobial activity of subgingival strains was comparable to the same species isolated from saliva (Table 10).

Thus, according to our data, lactobacilli expressed significant antimicrobial activity against periodontal pathogens, which enables them to maintain the balance of the oral microflora. Therefore, lactobacilli-preserving measures in the treatment of oral diseases should be preferred.

Table 10. Antimicrobial activity of oral lactobacilli originating from chronic periodontitis (CP) and periodontally healthy subjects, expressed as inhibition zone values (mm).

Lactobacilli		Inhibition of target bacteria: zone values (mm) mean ± SD				
Study group/ origin	Fermentation type / species	*S. mutans*	*A. actinomycetemcomitans*	*P. gingivalis*	*P. intermedia*	*C. albicans*
Healthy subjects						
Salivary	OHOL, *n* =	*19*	*19*	*15*	*15*	*19*
	L. acidophilus	0	2.5 ± 0	12.0 ± 0	0	0
	L. crispatus	0	1.0 ± 0.7	26.7 ± 0	9.5 ± 0	0
	L. delbrueckii	1.5 ± 0	4.3 ± 0	11.3 ± 0	0	0
	L. gasseri	0.1 ± 0.2*	1.5 ± 0.9	17.1 ± 4.0	6.7 ± 2.4	0
	L. salivarius	2.7 ± 2.1	4.2 ± 0.8	24.4 ± 4.2	11.2 ± 1.9	0
	FHEL, *n* =	*17*	*17*	*9*	*9*	*17*
	L. paracasei	2.2 ± 1.5	3.7 ± 1.2	24.0 ± 2.1	12.0 ± 1.9	0
	L. plantarum	3.0 ± 0.8	6.1 ± 0.7	21.7 ± 5.4	9.6 ± 1.3	0
	L. rhamnosus	2.0 ± 0.5	4.4 ± 1.0	22.1 ± 1.2	11.2 ± 0.3	0
	OHEL, *n* =	*24*	*24*	*12*	*12*	*24*
	L. fermentum	1.3 ± 0.8	3.1 ± 2.4	0	0	0
	L. oris	0.1 ± 0.4	2.4 ± 1.5	12.1 ± 5.7	0	0
Subgingival	OHOL, *n* =	*3*	*3*	*3*	*3*	*3*
	L. gasseri	0	1.8 ± 0.4	17.4 ± 5.1	4.5 ± 3.0	0
	FHEL, *n* =	*1*	*1*	*1*	*1*	*1*
	L. paracasei	2.5 ± 0	6.0 ± 0	23.0 ± 0	10.3 ± 0	0
	OHEL, *n*=	*3*	*3*	*2*	*2*	*3*
	L. oris	0	1.1 ± 1.9	7.4 ± 7.9	0	0
CP patients						
Salivary	OHOL, *n* =	*5*	*5*	*5*	*5*	*5*
	L. gasseri	1.5 ± 0.7*	0.5 ± 0.5	16.7 ± 1.9	8.5 ± 0.1	0
	L. salivarius	4.4 ± 1.2	4.0 ± 1.1	25.0 ± 5.6	13.5 ± 1.8	0
	FHEL, *n* =	*27*	*27*	*10*	*10*	*27*
	L. paracasei	2.0 ± 1.4	3.9 ± 0.9	19.9 ± 3.3	9.2 ± 1.9	0
	L. plantarum	3.7 ± 1.1	5.5 ± 1.8	21.1 ± 2.2	9.7 ± 0.9	0
	L. rhamnosus	2.3 ± 0.5	3.2 ± 0.8	24.2 ± 4.4	12.4 ± 1.9	0
	OHEL, *n* =	*16*	*16*	*6*	*6*	*16*
	L. fermentum	1.6 ± 0.6	2.0 ± 2.4	0	0	0
	L. oris	0	2.1 ± 0.6	14.0 ± 4.2	0	0

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.

Difference in the antimicrobial activity between salivary *L. gasseri* strains of healthy subjects (n=9) vs. salivary *L. gasseri* strains of CP patients (n=3): $^{*}P<0.05$.

16.3. Relationships between lactobacilli, periodontal pathogens and clinical parameters

The presence of the OHOL group of lactobacilli and especially *L. gasseri* was inversely associated with chronic periodontitis-related clinical parameters (Table 11). Their inverse associations were also seen with subgingival colonization of periodontal pathogens *A. actinomycetemcomitans*, *P. intermedia/ nigrescens* and *P. gingivalis* as well as with co-infection with at least two of the forenamed pathogens. No significant correlations were found for the other fermentation groups.

Health-promoting activity of homofermentative lactobacilli, including *L. gasseri*, has been noted in previous studies but not investigated in the case of oral diseases (Sakamoto *et al*., 2001). It has been shown that the strains in the OHOL group, in addition to antimicrobial activity, have high antioxidative ability, being useful for the host in reducing oxidative damage of human cells (Annuk *et al*., 2003; Lin and Yen, 1999; Kullisaar *et al*., 2003). Recently, Kitazawa *et al*. (2002) revealed a novel immunostimulating aspect of homofermentative lactobacilli as *L. acidophilus* and *L. gasseri* induced significant chemotaxis of macrophages.

Thus, the presence of lactobacilli with antimicrobial activity as well as with good antioxidative and immunostimultative properties could be one of the factors regulating the presence and the number of periodontal pathogens.

Table 11. Correlations between the presence of certain fermentation types of lactobacilli, and clinical parameters and presence of subgingival periodontal pathogens.

Periodontal status	Fermentation type[1] /species[2] of lactobacilli	Correlation coefficient R	*P*-value
Plaque index (PI)	vs. OHOL	–0.38	0.032
	vs. *L. gasseri*	–0.44	0.045
	vs. FHEL	0.09	NS
	vs. OHEL	–0.26	NS
Gingival index (GI)	vs. OHOL	–0.40	0.023
	vs. *L. gasseri*	–0.49	0.025
	vs. FHEL	0.11	NS
	vs. OHEL	–0.30	NS
Periodontal probing depth (PPD)	vs. OHOL	–0.40	0.027
	vs. *L. gasseri*	–0.46	0.039
	vs. FHEL	0.17	NS
	vs. OHEL	–0.13	NS

Table 11. (Continuation)

Periodontal pathogens	Fermentation type[1] /species[2] of lactobacilli	Correlation coefficient R	*P*-value
A. actinomycetemcomitans	vs. OHOL	–0.35	0.040
	vs. *L. gasseri*	–0.43	0.038
	vs. FHEL	0.18	NS
	vs. OHEL	–0.10	NS
P. intermedia / nigrescens	vs. OHOL	–0.40	0.019
	vs. *L. gasseri*	–0.40	NS
	vs. FHEL	0.01	NS
	vs. OHEL	–0.19	NS
P. gingivalis	vs. OHOL	–0.26	NS
	vs. *L. gasseri*	–0.38	NS
	vs. FHEL	0.10	NS
	vs. OHEL	–0.08	NS
At least 2 pathogens	vs. OHOL	–0.35	0.040
	vs. *L. gasseri*	–0.48	0.019
	vs. FHEL	0.18	NS
	vs. OHEL	–0.10	NS

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative;
NS, not statistically significant.
[1]Data given for all subjects.
[2]Data given for 23 subjects.

17. Use of oral lactobacilli for prevention and treatment of oral diseases (Papers IV, VI)

17.1. Selection of oral lactobacilli for use as probiotics

As the next step of the study, we attempted to select the lactobacilli strains with the potential to be used as probiotics against oral diseases. Although lactobacilli are generally regarded as safe (GRAS), several requirements have been proposed for novel probiotic strains (Saarela *et al*., 2000). According to these requirements, various properties were tested for our lactobacilli strains.

17.1.1. Origin and species composition

Isolates from healthy humans are advised (Saarela *et al*., 2000), and therefore only the strains originating from periodontally healthy subjects (60 salivary, 7 subgingival) were included in further study. As there has been observed significant fermentation type-, species- and strain-specific variability in the acid and bile tolerance as well as in the antimicrobial activity of lactobacilli (Jacobsen *et al*., 1999; Annuk *et al*., 2003), several strains from various fermentation types and species should be tested to choose the best ones. The set of the strains studied by us were in line with these suggestions: they contained all three fermentation types (22 OHOL, 18 FHEL, 27 OHEL) and ten species (Table 12 and 13).

17.1.2. Antimicrobial activity

Good antimicrobial properties of probiotic strains play a key role in eradicating or inhibiting pathogenic and opportunistic bacteria. The majority of *Lactobacillus* strains of periodontally healthy persons suppressed the growth of *A. actinomycetemcomitans* (89% of tested strains), *P. gingivalis* (83%), *P. intermedia* (62%) and *S. mutans* (55%). The antimicrobial activity was mainly species-specific as described above (see 16.2.). The highest antimicrobial activity was associated with facultatively heterofermentative *L. paracasei*, *L. plantarum* and *L. rhamnosus*, and homofermentative *L. salivarius* (Table 10). In addition, homofermentative *L. crispatus* and *L. gasseri* showed quite high activity against anaerobic *P. gingivalis* and *P. intermedia*.

17.1.3. Tolerance of environmental factors

In order to survive in and colonize the digestive tract, the probiotic bacteria must survive passage through the acidic oral and gastric environment and tolerate the effects of bile produced by the small intestine of humans (Ouwehand *et al*., 1999).

17.1.3.1. Acid tolerance

Acid tolerance by lactobacilli was found to be strain-, species- and fermentation type-specific (Table 12). Nearly all strains (65 of 67) survived for 4 h at pH 3.0 but only 28 were able to grow at this pH. Survival following 4 h of incubation at pH 2.5 was observed for 25 (10 FHEL, 15 OHEL strains) of 31 strains but none of the strains tested grew at this pH, confirming the results by Jacobsen *et al*. (1999). Heterofermentative *L. plantarum* and *L. fermentum* were the most

tolerant species. The acid tolerance of subgingival strains was comparable to the same species isolated from saliva.

The pH of the stomach may fall as low as 1.0, but when food comes into the stomach, pH may rise to levels of 3.0 to 4.0 level due to the buffering capacity of proteins. In resting dental plaque, pH has been reported to be around 6.5 (ranging between 5.6 and 7.0), with a drop down to 4.5 and 4.0 following a sucrose rinse (Nyvad and Fejerskov, 1994). Most of the studied strains resisted incubation at pH 3.0 for 4 h, which makes them good candidates for use as probiotics.

Table 12. Survival and growth of lactobacilli in an acidic environment, expressed as a percentage (%) of surviving and growing strains.

Lactobacilli			Growth[1] of strains (%) at pH			Survival[2] of strains (%) at pH						
			5.6	3.5	3.0[§]	5.6	3.5	3.0		2.5	2.0	1.5
Origin	Fermentation type[1]/ species	No. of strains tested							No. of strains tested			
Salivary	OHOL											
	L. acidophilus	*2*	100	0	0	100	100	100				
	L. crispatus	*2*	100	0	0	100	100	100				
	L. delbrueckii	*1*	100	0	0	100	0	0				
	L. gasseri	*9*	100	22	0	100	100	89	*1*	0	0	0
	L. salivarius	*5*	100	80	0	100	100	100	*3*	0	0	0
	FHEL											
	L. paracasei	*7*	100	86	29	100	100	100	*1*	100	0	0
	L. plantarum	*7*	100	100	100	100	100	100	*7*	100	0	0
	L. rhamnosus	*3*	100	100	33	100	100	100	*3*	67	0	0
	OHEL											
	L. fermentum	*16*	100	100	100	100	100	100	*15*	100	0	0
	L. oris	*8*	100	100	25	100	100	100				
Sub-gingival												
	L. gasseri	*3*	100	0	0	100	100	100				
	L. paracasei	*1*	100	100	0	100	100	100	*1*	0	0	0
	L. oris	*3*	100	100	0	100	100	100				

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.

[1]Data presented following incubation for 24 hours: [§], no growth was observed at pH lower than 3.0.

[2]Data presented following incubation for 4 hours.

17.1.3.2. Bile tolerance

The tested strains showed relatively high tolerance of bile salts: half of the strains (11 OHOL, 14 FHEL, 8 OHEL) were able to grow at bile concentration of 5% (v/v) following 24 h of incubation (Table 13). The most tolerant species were heterofermentative *L. paracasei* and *L. rhamnosus*, and homofermentative *L. acidophilus*. The bile tolerance of subgingival strains was comparable to the same species isolated from saliva.

Bile content (v/v) in the small intestine is around 0.5 to 2%. In our study most of the strains grew well at these bile concentrations, indicating that oral lactobacilli have the potentiality to be used as probiotics in the gastrointestinal tract as well. However, in this case additional tests are needed, such as adherence to human intestinal cells and antimicrobial activity against intestinal pathogens.

Table 13. Growth of lactobacilli at various concentrations of bile following incubation for 24 h, expressed as a percentage (%) of growing strains.

Lactobacilli			Growth of strains (%) at bile concentration (% v/v)						
Origin	Fermentation type/species	No. of strains tested	0.08	0.16	0.3	0.6	1.25	2.5	5
Salivary	OHOL								
	L. acidophilus	*2*	100	100	100	100	100	100	100
	L. crispatus	*2*	100	100	100	100	100	50	0
	L. delbrueckii	*1*	100	100	100	100	100	100	0
	L. gasseri	*9*	100	100	100	89	89	78	56
	L. salivarius	*5*	100	100	100	100	100	100	60
	FHEL								
	L. paracasei	*7*	100	100	100	100	100	100	100
	L. plantarum	*7*	100	100	100	100	100	100	43
	L. rhamnosus	*3*	100	100	100	100	100	100	100
	OHEL								
	L. fermentum	*16*	100	100	100	100	94	19	0
	L. oris	*8*	100	100	100	100	100	100	75
Subgingival									
	L. gasseri	*3*	100	100	100	100	67	33	33
	L. paracasei	*1*	100	100	100	100	100	100	100
	L. oris	*3*	100	100	100	100	100	100	67

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.

Table 14. Antimicrobial activity, acid and bile tolerance of 22 selected salivary and subgingival *Lactobacillus* strains.

Lactobacilli			Antimicrobial activity toward					Survival[3] at	Growth[4] at		Score[5]
Origin	Strain	Species	Sm[1]	Aa[1]	Pg[2]	Pi[2]	Ca[1]	pH	pH	bile (% v/v)	
Saliva	25-DLB-3A-A	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	27-DLB-1	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	35-DLB-2	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	21-DLB-4-B	*L. paracasei*	+	++	++	+	–	3.0[§]	3.0	5	16
	37-DLB-2A	*L. plantarum*	+	++	+	+	–	2.5	3.0	5	15
	27-DLB-2A	*L. salivarius*	++	+	++	+	–	3.0	3.5	2.5	15
	43-DLB-3-A	*L. salivarius*	+	++	++	+	–	3.0	3.5	5	15
	21-DLB-5-B	*L. rhamnosus*	+	++	++	+	–	2.5	3.5	5	15
	35-DLB-5	*L. rhamnosus*	–	++	++	+	–	3.0	3.0	5	15
	21-DLB-6-B	*L. paracasei*	+	+	++	+	–	3.0[§]	3.5	5	14
	37-DLB-1	*L. salivarius*	+	+	++	+	–	3.0	3.5	5	14
	21-DLB-7	*L. rhamnosus*	+	+	++	+	–	2.5	3.5	5	14
	13-DLB-6A	*L. gasseri*	–	+	++	+	–	3.0[§]	3.5	1.25	11
	33-DLB-2	*L. gasseri*	–	+	+	+	–	3.0	3.5	5	11
	13-DLB-4A	*L. fermentum*	+	++	–	–	–	2.5	3.0	1.25	10
	37-DLB-2B	*L. fermentum*	–	++	–	–	–	2.5	3.0	2.5	10
	21-DLB-1B-2	*L. oris*	–	+	+	–	–	3.0[§]	3.0	5	10
	25-DLB-4A-1	*L. oris*	–	++	+	–	–	3.0[§]	3.5	5	10
Sub-gingival	8-2-16A-B	*L. paracasei*	+	++	++	+	–	3.0	3.5	5	15
	8-2-1A	*L. gasseri*	–	–	+	+	–	3.0[§]	5.6	5	9
	37-2-10-A	*L. gasseri*	–	+	++	–	–	3.0[§]	5.6	1.25	9
	8-2-16B	*L. oris*	–	+	+	–	–	3.0[§]	3.5	5	9

[1]Antimicrobial activity: –, no inhibition or less than 2mm; +, between 2 and 5 mm of inhibition; ++, 5 mm of inhibition and above; Sm, *S. mutans*; Aa, *A. actinomycetemcomitans*; Ca, *C. albicans*.
[2]Antimicrobial activity: –, no inhibition or less than 7 mm; +, between 7 and 20 mm of inhibition; ++, 20 mm of inhibition and above; Pg, *P. gingivalis*; Pi, *P. intermedia*.
[3]Data presented following incubation for 4 hours: [§], not determined at pH lower than 3.0.
[4]Data presented following incubation for 24 hours.
[5]Total score for a *Lactobacillus* strain based on the data of antimicrobial activity, acid and bile tolerance (max. value 19).

17.1.4. Selection of the best strains

Each particular *Lactobacillus* strain was scored according to the results of antimicrobial activity (score 0–3 for each target microorganism), acid (score 0–2) and bile tolerance (score 0–2) with a maximum value of 19 (Paper VI). Based on the scoring results we selected 22 (18 salivary and 4 subgingival) most promising lactobacilli for probiotic use (Table 14). The strains belonging to species of *L. plantarum*, *L. paracasei*, *L. salivarius* and *L. rhamnosus* showed both good antimicrobial activity and good tolerance of low pH and high concentration of bile.

17.1.5. Susceptibility to antibiotics

An important requirement for probiotic strains is that they should not carry transferable antibiotic resistance genes. The spread of such genes among bacterial species through lateral gene transfer may contribute to dissemination of resistance to antibiotics used for therapy (Saarela *et al*., 2000; European Commission, 2001; Duncan, 2003). Therefore, twenty-two most promising lactobacilli for probiotic use were screened for a group of 13 antibiotics having a different mechanism of action.

We found that oral lactobacilli, as have also been observed for intestinal lactobacilli (Mändar *et al*., 2001), did not display uniform susceptibility to antibiotics. No resistance was found to amoxicillin, cefprozil, cefotaxime, erythromycin and chloramphenicol (Table 15). Although most of the strains had low MICs to gentamicin, doxycycline, tetracycline and clindamycin, some resistant strains appeared. One strain of *L. gasseri* was resistant to gentamicin, all 4 *L. plantarum* and 2 *L. oris* strains, to doxycycline and/or tetracycline and all 4 *L. gasseri* strains, to clindamycin. All studied lactobacilli were resistant to metronidazole and majority of the strains, belonging to different species, were resistant to cefoxitin, vancomycin and ciprofloxacin. All vancomycin susceptible strains belonged to *L. gasseri,* while cefoxitin and ciprofloxacin susceptible strains belonged to species of *L. gasseri*, *L. salivarius* and *L. paracasei*. When testing for ciprofloxacin (2 *L. salivarius*, 1 *L. paracasei* and 2 *L. rhamnosus* strains), cefotaxime (2 *L. oris*) and cefprozil (2 *L. fermentum*), pinpoint colonies were observed within the inhibition zone of the E-test. Following retesting of the strains, these colonies were considered as resistant mutants and were included in the MICs.

It is important to confirm whether the antibiotic resistance of a probiotic strain is of intrinsic origin or is carried by highly mobile genetic elements, such as plasmids and transposons (Saarela *et al*., 2000). High resistance to metronidazole, cefoxitin, vancomycin and ciprofloxacin has been reported previously (Hamilton-Miller and Shah, 1998; Mändar *et al*., 2001; Danielsen and Wind,

Table 15. Antibiotic susceptibility of 22 selected salivary and subgingival *Lactobacillus* strains.

Lactobacilli		Antibiotic with the MIC (μg/ml) as follows:												
Strain	Species	AC	FX	FP	CT	VA	GM	EM	DC	TC	CM	CL	CI	MZ
Salivary strains														
25-DLB-3A-A	*L. plantarum*	0.25	≥256	0.5	0.25	≥256	2	0.38	16	24	0.38	3	≥32	≥256
27-DLB-1	*L. plantarum*	0.38	≥256	0.5	0.38	≥256	1.5	0.5	24	24	0.25	4	≥32	≥256
35-DLB-2	*L. plantarum*	0.19	≥256	0.38	0.25	≥256	1.5	0.25	6	24	1.5	4	≥32	≥256
21-DLB-4-B	*L. paracasei*	1.0	≥256	6	6	≥256	4	0.19	0.75	0.75	0.09	3	2	≥256
37-DLB-2A	*L. plantarum*	0.12	≥256	0.38	0.19	≥256	1.0	0.5	16	32	0.75	4	≥32	≥256
27-DLB-2A	*L. salivarius*	0.75	16	1.5	0.75	≥256	12	0.75	1.0	1.5	0.25	2	1.5	≥256
43-DLB-3-A	*L. salivarius*	0.19	1.5	0.75	0.25	≥256	2	0.25	0.25	0.25	0.19	1.5	≥32	≥256
21-DLB-5-B	*L. rhamnosus*	0.5	≥256	6	4	≥256	8	0.38	0.5	1.0	0.38	4	12	≥256
35-DLB-5	*L. rhamnosus*	0.75	≥256	8	6	≥256	12	0.25	0.5	0.5	0.75	3	4	≥256
21-DLB-6-B	*L. paracasei*	0.75	≥256	6	6	≥256	4	0.19	0.75	0.75	0.25	3	6	≥256
37-DLB-1	*L. salivarius*	0.25	6	0.75	0.38	≥256	12	0.38	0.75	1.0	0.19	2	≥32	≥256
21-DLB-7	*L. rhamnosus*	0.75	≥256	8	6	≥256	8	0.25	0.5	0.5	0.5	3	12	≥256
13-DLB-6A	*L. gasseri*	0.38	≥256	3	1.0	1.5	8	0.19	2	3	8	6	≥32	≥256
33-DLB-2	*L. gasseri*	0.5	≥256	1.5	2	1.5	12	0.09	1.5	0.75	8	3	≥32	≥256
13-DLB-4A	*L. fermentum*	0.25	≥256	12	0.5	≥256	1.5	0.12	3	2	0.01	3	≥32	≥256
37-DLB-2B	*L. fermentum*	0.25	128	0.75	0.5	≥256	0.25	0.09	6	6	0.02	3	6	≥256
21-DLB-1B-2	*L. oris*	0.38	≥256	3	1.5	≥256	2	0.19	16	24	0.03	6	≥32	≥256
25-DLB-4A-1	*L. oris*	0.25	≥256	4	1.5	≥256	0.75	0.19	12	16	0.12	4	≥32	≥256
Subgingival strains														
8-2-16A-B	*L. paracasei*	1.0	≥256	8	12	≥256	8	0.25	0.75	0.75	0.25	3	1.0	≥256
8-2-1A	*L. gasseri*	0.19	32	1.5	0.38	1.5	16	0.19	1.5	1.5	48	3	≥32	≥256
37-2-10-A	*L. gasseri*	0.19	4	1.5	0.5	1.5	6	0.12	0.38	0.38	4	2	≥32	≥256
8-2-16B	*L. oris*	0.38	≥256	2	0.38	≥256	0.25	0.12	12	12	0.19	3	≥32	≥256

AC, amoxicillin; FX, cefoxitin; FP, cefprozil; CT, cefotaxime; VA, vancomycin; GM, gentamicin; EM, erythromycin; DC, doxycycline; TC, tetracycline; CM, clindamycin; CL, chloramphenicol; CI, ciprofloxacin; MZ, metronidazole.

2003) and could be interpreted as high natural resistance to these antibiotics. On the other hand, plasmid-encoded erythromycin and tetracycline resistance has been reported for lactobacilli (Axelsson *et al*., 1988; Gevers *et al*., 2003) and therefore, tetracycline resistant strains of *L. plantarum* and *L. oris* should be further tested by genetic analysis and transfer experiments in order to determine whether they have transferable resistance genes. If not, moderate natural resistance could be favorable when used in antibiotic/probiotic combination therapies. The additional value of this is study is to reveal the susceptibility pattern of oral lactobacilli, which enables one to avoid antibiotics that may damage the beneficial lactoflora.

Hence, based on thorough testing, including exact identification, detection of antimicrobial activity, resistance to environmental factors and antibiotic susceptibility pattern, we have selected a couple of strains that have the potentiality to be used as first generation probiotics as well as for development of second generation probiotics.

17.2. Development of a second generation probiotic by the expression of single-chain antibody against the periodontal pathogen *P. gingivalis* in *Lactobacillus*

In parallel with the testing of the lactobacilli strains of periodontally healthy subjects, aimed to be selected and used as suitable probiotics, we attempted to develop a second generation probiotic using *L. paracasei* ATCC 393 as the model.

In previous reports the monoclonal antibody 61BG1.3 has been reported to confer protection against recolonization with *P. gingivalis* in humans (Booth *et al.,* 1996). In this study, we have expressed a functional single chain antibody fragment (scFv) derived from the monoclonal antibody 61BG1.3, which is directed against the arginine-specific protease of *P. gingivalis* (RgpA) on the surface of *Lactobacillus*.

17.2.1. Construction of the *Lactobacillus* expressing surface scFv against RgpA

The scFv-encoding gene derived from the IgG1 monoclonal antibody 61BG1.3 was fused to an E-tag-encoding sequence and cloned in the plasmid pLP501 (Fig. 2). The scFv-containing plasmid, named pLP501-scFv(61BG1.3)-long anchor, was introduced into *L. paracasei* in order to generate lactobacilli producing scFv antibody fragments against the RgpA protease on their surface. In pLP501, the scFv expression is under the control of the constitutive promoter of the lactate dehydrogenase gene (Fig. 2).

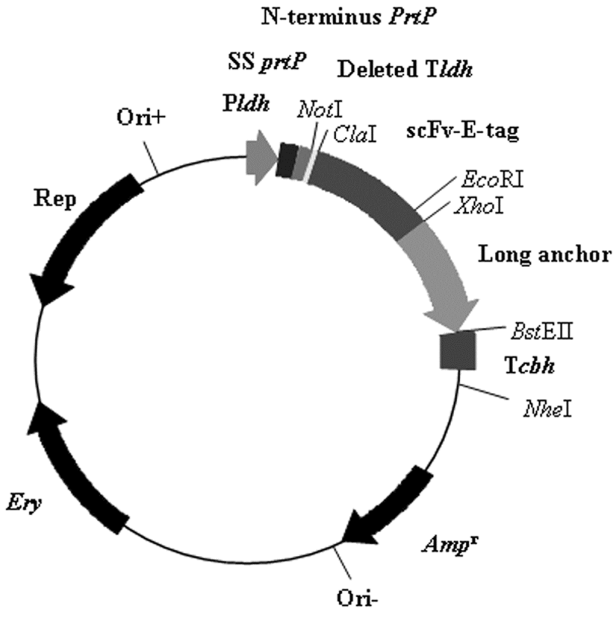

Figure 2. Map of the *Lactobacillus* pLP501-scFv(61BG1.3)-long anchor vector. The vector mediates surface-anchored expression of scFv by fusion to the last 244 amino acids of *L. casei* proteinase P. P*ldh*, promotor sequence of the lactate dehydrogenase gene of *L. casei*; SS *prtP*, signal sequence of the *PrtP* gene (33 amino acids); N-terminus *prtP*, N-terminus (36 amino acids) of the *PrtP* gene; T*ldh*, transcription terminator of the lactate dehydrogenase gene of *L. casei*; deleted T*ldh*, remaining sequence after deletion of T*ldh*; scFv, single-chain antibody against RgpA proteinase; long anchor, anchor sequence from the proteinase P gene of *L. casei* (244 amino acids); T*cbh*, transcription terminator sequence of the conjugated bile acid hydrolase gene of *L. plantarum* 80; *Amp*^{r}, ampicillin-resistance gene; *Ery*, erythromycin-resistance gene; Rep, *repA* gene of plasmid p353-2 from *L. pentosis*; Ori, origin of replication (Ori+ = ori *E. coli*, Ori- = ori *Lactobacillus*). Reprinted with permission.

17.2.2. Analysis of the expression of the scFv

Surface expression of the scFv on lactobacilli transformed with pLP501-scFv(61BG1.3)-long anchor and pLP501-scFv-long anchor (scFv antibody directed against the surface adhesion antigen SAI/II of *S. mutans*), was analysed by flow cytometry using an anti-E-tag antibody. The pLP501-scFv(61BG1.3)-long anchor–transformed lactobacilli showed a positive signal when stained by the anti-E-tag antibody, but slightly lower than the lactobacilli transformed with pLP501-scFv–long anchor (Fig. 3). The amount of scFv anti-RgpA expressed on the surface of lactobacilli was estimated by densitometry using a standard curve of an affinity purified E-tagged scFv and showed approximately 8 ng per 10^8 bacteria or 850 scFv fusion molecules/bacterium.

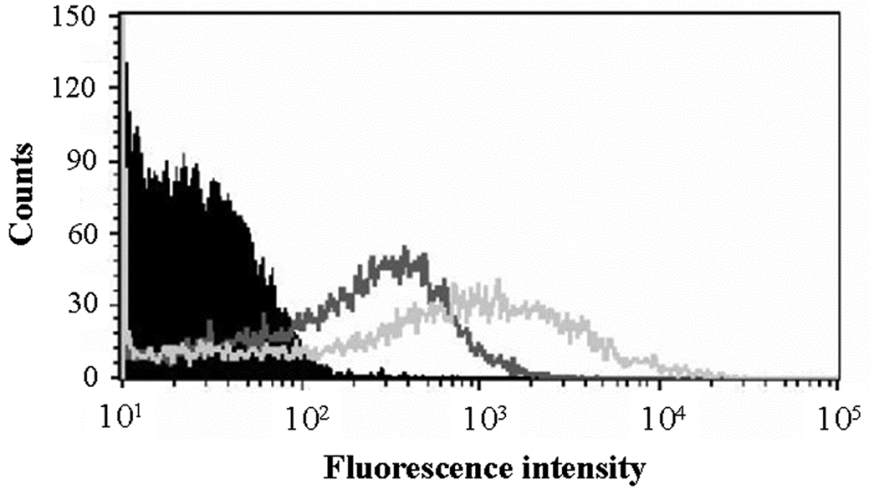

Figure 3. Flow cytometry analysis showing the expression of scFv (61BG1.3) on the surface of *L. paracasei* by detection of the E-tag by a mouse anti-E-tag antibody. Black filled curve, non transformed lactobacilli; dark grey line, *L. paracasei* expressing a scFv (61BG1.3) against RgpA; light grey line, *L. paracasei* expressing a scFv against the SAI/II adhesin. Reprinted with permission.

17.2.3. Biological activity of the scFv

The scFv extracted from *Lactobacillus* was added to plates coated with purified $(His)_6$ tag RgpA or *S. mutans* SAI/II antigen (negative control). The scFv anti-RgpA extracted from the transformed *L. paracasei* or intact recombinant bacteria bound to RgpA in ELISA but not to SAI/II antigens (Table 16). Extract or whole cells of *Lactobacillus* expressing scFv anti-SAI/II bound to SAI/II antigen. The monoclonal antibodies 61BG1.3 and Guy's 13 bound to RgpA and SAI/II, respectively. The concentration of scFv anti-RgpA in the bacterial extract used in ELISA was about 200 ng/ml. The reactivity of the scFv anti-RgpA was nearly 20 times lower than the corresponding anti-RgpA monoclonal antibody 61BG1.3. Considering that the molecular weight of the fusion protein is 2.4 times smaller than an IgG1 molecule, we estimate that the reactivity of the scFv is approximately 50 times lower than the parent bivalent monoclonal antibody. This may be because of either the monovalency of the scFv resulting in low avidity or because of improper folding of the protein.

Agglutination assay showed the presence of visible aggregates when mixing *L. paracasei* pLP501-scFv(61BG1.3)-long anchor with *P. gingivalis* W83 whereas no agglutination was observed when mixing *P. gingivalis* with pLP501-scFv-long anchor (anti-SAI/II) or non-transformed lactobacilli. No autoaggregation was seen when testing *L. paracasei* pLP501-scFv(61BG1.3)-long anchor or *P. gingivalis* alone. These results were confirmed by Gram staining of the

slides, which also revealed the presence of aggregates containing *L. paracasei* pLP501-scFv(61BG1.3)-long anchor and *P. gingivalis* W83 (Fig. 4). Scanning electron microscopy also showed binding of *P. gingivalis* to *Lactobacillus* expressing the scFv against *P. gingivalis*.

Table 16. Binding activity of the scFv to RgpA

	OD 405 nm	
	RgpA coating	SAI/II coating (control)
Cell extracts[1]		
pLP501-scFv(61BG1.3)-long anchor (anti-RgpA)	0.195	0.084
Non-transformed	0.082	0.080
pLP501-scFv-long anchor (anti-SAI/II)	0.085	0.205
Whole bacteria[2]		
pLP501-scFv(61BG1.3)-long anchor (anti-RgpA)	0.133	0.085
Non-transformed	0.087	0.083
pLP501-scFv-long anchor (anti-SAI/II)	0.082	0.145
Monoclonal antibody 61BG1.3 (anti-RgpA)		
250 ng/ml	1.508	0.078
25 ng/ml	0.316	0.075
2.5 ng/ml	0.122	0.079
Monoclonal antibody Guy's 13 (anti-SAI/II)		
100 ng/ml	0.076	1.875
10 ng/ml	0.077	0.434
1 ng/ml	0.079	0.138

[1]Bacteria were disrupted, centrifuged and the supernatant subsequently added to RgpA or SAI/II coated wells. The concentration of scFv anti-RgpA in extract was estimated to approximately 200 ng/ml.
[2]Bacteria were added at a concentration of 10^8 bacteria/ml.

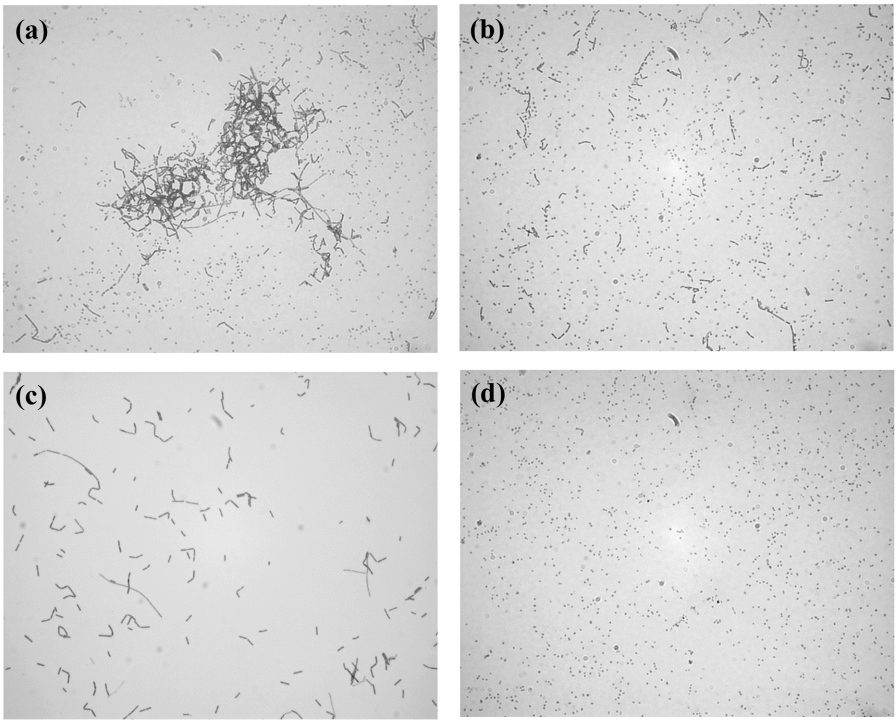

Figure 4. Gram staining of agglutination assay with (a) *L. paracasei* expressing a scFv (61BG1.3) against RgpA and *P. gingivalis*, (b) non-transformed lactobacilli and *P. gingivalis*, (c) *L. paracasei* expressing a scFv against RgpA only, (d) *P. gingivalis* only. Reprinted with permission.

17.2.4. Antimicrobial activity of the construct

Non-transformed lactobacilli and lactobacilli transformed with pLP501-scFv(61BG1.3)-long anchor were both shown to equally inhibit the growth of *P. gingivalis* W83 on agar plates, suggesting that expression of scFv by lactobacilli does not affect its bacteriostatic/bacteriocidal activities. The mean width of inhibition zone was 7.3 ± 0.2 mm mm for the non-transformed lactobacilli and 7.6 ± 0.4 mm for the transformed one.

GENERAL DISCUSSION

18. Altered subgingival microbial ecology in chronic periodontitis

An abundant mixed aerobic and anaerobic bacterial flora was found in the periodontal pockets of chronic periodontitis patients. The most frequently isolated bacteria were various anaerobic gram-negative rods, coryneforms, streptococci, peptostreptococci and veillonella. Large inter-individual variations in the number of different species and their counts were seen. This clearly supports the data on the individuality of a host's microflora, assessed in different biotopes of gastrointestinal and urogenital tract (Mikelsaar *et al*., 2004). As expected and in agreement with previous reports (Zambon, 1996; Ximénez-Fyvie *et al*., 2000a), the mean total count of microorganisms was much higher in the periodontitis pockets as compared with the healthy crevices. This could be explained by different subgingival environmental conditions and with an obviously larger area for bacterial colonization in the periodontal pockets. In addition, the high level of inflammation in diseased sites probably increased the amount of necessary nutrients for bacterial multiplication. Moreover, the possible mechanisms aiming to keep the bacterial numbers under control might have weakened.

In the present study, along with the increase in the absolute numbers of microbes, the number of acknowledged periodontal pathogens (*P. gingivalis*, *P. intermedia/nigrescence*, *A. actinomycetemcomitans*) also increased in periodontitis, yet the prevalence of *P. gingivalis* remained lower than that noted in some earlier reports (Söder *et al*., 1993; van Winkelhoff *et al*., 2002). This could be explained by the differences in methodology, but recently the regional differences in the composition of the subgingival microbiota have been revealed as well (Haffajee *et al*., 2004). We also observed that periodontally healthy persons were never colonized with *A. actinomyctemcomitans* and *P. gingivalis*, while the colonization with *P. intermedia*/*nigrescens* was seen in half of them. Co-infection with two or three pathogens was seen in nearly half of the periodontitis patients, but was not observed in healthy subjects. These data support the idea (Söder *et al*., 1993; Socransky *et al*., 1998) that the mere presence of a single periodontal pathogen does not necessarily lead to periodontal destruction. Instead, the possibility of causing inflammation may be dependent on the particular combination of various microorganisms.

In further analysis, the proportions (%) of bacteria in the total count of microorganisms in a particular periodontal site were calculated. As the absolute count of bacterial species varies greatly in different individuals, proportional analysis evens up such individual differences and is more informative (Mikelsaar *et al*., 2004). In agreement with some previous findings (Slots 1977a,b; Moore and Moore, 1994; Ximénez-Fyvie *et al*., 2000a), a significant imbalance

in the quantitative composition of the periodontal microflora occurred in chronic periodontitis patients with a predominance of anaerobic microorganisms by respiration type and gram-negative microorganisms by structure of cell wall, whereas aerobic and gram-positive species predominated in healthy individuals. The significant increase in the proportion of different anaerobic gram-negative rods in diseased sites occurred mostly at the expense of certain aerobic gram-positive bacteria, since periodontally healthy patients harbored much higher proportions of streptococci and aerobic coryneforms in their subgingival sites.

As we also revealed remarkable antimicrobial properties of the predominant lactic acid producing streptococci, the idea that lactic acid bacteria might play an important role in maintenance of a balanced oral microenvironment, was proved during the subsequent extended investigation of oral lactobacilli.

19. The role of oral lactic acid bacteria in chronic periodontitis

Investigating the pathogenesis of chronic periodontitis, it is generally accepted that the disease is caused by bacteria in dental plaque, with evidence that specific periodontal pathogens are responsible for the development of the disease. However, as shown in the present study and in previous studies, some individuals may harbor these periodontal pathogens, but do not appear to show clinical evidence of disease (Griffen *et al*., 1998; van Winkelhoff *et al*., 2002). These data suggest that the virulence of particular strains might be different (Griffen *et al*., 1999) or their numbers are kept below the detrimental threshold for the host by the other members of the indigenous microflora. In the present study, we found that in comparison to patients with chronic periodontitis, periodontally healthy subjects had a higher proportion of lactic acid bacteria in their subgingival microflora. Moreover, we showed for the first time that the composition of the *Lactobacillus* species in the oral cavity differed with respect to periodontal health. Further, most of the oral lactobacilli, both homo- and hetereofermentatives, as well as *S. mutans* were able to inhibit the growth of putative periodontal pathogens *in vitro*. These findings point to the periodontal health-promoting role of oral lactic acid microorganisms.

In previous studies, various *Streptococcus* (e.g. *S. sanguis*, *S. oralis*, *S. gordonii*) and coryneform (e.g. *A. naeslundii*, *A. odontolyticus*) species have been associated with gingival health (Tanner *et al*., 1996; Ali *et al*., 1997; Darveau *et al*., 1997; Ximénez-Fyvie *et al*., 2000a). Similar results were obtained in the present study where we found a significantly higher proportion of streptococci, particularly *S. mutans* and *S. mitis* groups, as well as aerobic coryneforms (e.g. *Actinomyces* sp.) in periodontally healthy persons as compared with chronic periodontitis patients. Our major finding, which has

never been described before, was the significantly higher prevalence of obligately homofermentative lactobacilli, especially *L. gasseri*, among periodontally healthy persons. This suggests that homofermentative lactobacilli may serve as both indicators and promoters of periodontal health. Health-promoting activity of homofermentative lactobacilli has been noted in relation to gastrointestinal diseases; for example, Sakamoto *et al*. (2001) reported the effectiveness of *L. gasseri* in both suppressing *Helicobacter pylori* and reducing the gastric mucosal inflammation. In our study, all the above-mentioned lactic acid bacteria (homofermentative lactobacilli, including *L. gasseri*, as well as streptococci and aerobic coryneforms) were positively associated with periodontal health and inversely associated with periodontal pathogens.

Bacterial interactions, including antagonism, are likely to play an important role in the ecology of the oral microflora (Marcotte and Lavoie, 1998). Several inhibitory substances have been identified, including hydrogen peroxide, organic fatty acids, lactic acid, antibiotics, enzymes and bacteriocins. Inhibition of the *in vitro* growth of periodontal pathogens by viridans streptococci due to the production of hydrogen peroxide (Hillman *et al*., 1985) and antimicrobial activity of *Lactobacillus* and *Actinomyces* strains due to the production of various organic acids has been reported (Sookkhee *et al*., 2001; Tompkins and Tagg, 1986). Recently, Doran *et al*. (2004) showed that anaerobic bacteria (including *P. intermedia* and *P. gingivalis*) were inhibited by metabolic end products of glucose fermentation by oral streptococci, particularly strains of *S. mutans* and *S. salivarius*. We succeeded in confirming the ability of both *S. mutans* and lactobacilli to inhibit the growth of periodontal pathogens by our *in vitro* experiments. Testing more than one hundred lactobacilli strains belonging to ten species, we found that most *Lactobacillus* species were able to suppress the anaerobic *P. gingivalis* and *P. intermedia* and the microaerophilic *A. actinomycetemcomitans*. Among others, two species most frequently present in periodontally healthy subjects, *L. gasseri* and *L. fermentum*, also expressed high antimicrobial activity, but their targets were different: the former was more active against anaerobes, while the latter was more active against microaerophiles. At the same time, none of the periodontal pathogens was able to inhibit the growth of lactobacilli.

In addition to antimicrobial activity, homofermentative lactobacilli have been shown to express high antioxidative activity and, hence, they could be useful for the host in reducing oxidative damage of human cells (Annuk *et al*., 2003; Lin and Yen, 1999; Kullisaar *et al*., 2003). Recently, Kitazawa *et al*. (2002) revealed a novel immunostimulating aspect of homofermentative lactobacilli as *L. acidophilus* and *L. gasseri* induced significant chemotaxis of macrophages. Thus, the presence of homofermentative lactobacilli with antimicrobial activity as well as with good antioxidative and immunostimultative properties could be one of the factors regulating the presence and the number of periodontal pathogens. Therefore, lactobacilli-preserving measures in the treatment of oral diseases should be preferred. Antibiotics, delivered either locally or

systemically, are used as a valuable adjunct to mechanical therapy by many clinicians since major pathogens may escape from treatment due to their ability to invade the periodontal tissues (Meyer *et al*., 1991; Lamont *et al*., 1995), or because they reside at sites inaccessible to periodontal instruments. The most frequently used antibiotics include inhibitors of cell wall synthesis (e.g. penicillin, amoxicillin), protein synthesis (e.g. tetracycline, doxycycline, clindamycin) and nucleic acid synthesis (e.g. ciprofloxacin, metronidazole). In the present study, we found high natural resistance to ciprofloxacin and metronidazole, whereas most strains were sensitive to other antibiotics used in periodontal therapy.

Although our studies have indicated the beneficial role of lactic acid bacteria in oral microecology, representatives of this group, as mutans streptococci and lactobacilli, have been associated with dental caries. However, their association with caries is not unique as caries can occur in the apparent absence of these species, and on the contrary, mutans streptococci and lactobacilli can persist without evidence of detectable deminaralization (Marsh, 2003). In the present study, we investigated salivary lactobacilli in 96 schoolchildren and observed that lactobacilli colonized both caries-free and caries-active children, which confirms the results by Toi *et al*. (2000). Therefore, the presence of lactic acid bacteria may not be directly related to caries risk if their species composition or the virulence of a particular strain is unknown. In addition, the results of the present study showed that complex interactions may occur not only between lactic acid bacteria and putative periodontal pathogens but also between different species within the lactic acid group of bacteria, since oral lactobacilli were able to inhibit *S. mutans in vitro.* Previous studies in children and young adults have shown that consumption of milk products containing *L. rhamnosus* GG reduced the number of *S. mutans* and risk for dental caries (Näse *et al*., 2001; Ahola *et al*., 2002). Interestingly, we also found that as a whole the *Lactobacillus* strains isolated from chronic periodontitis patients were more active against *S. mutans* and our finding may at least partly explain an inverse association between dental caries and periodontal diseases observed by Sioson *et al*. (2000). These findings illustrate the complex and diverse microecological interrelations between oral indigenous and potentially patogenic bacteria, directed to balance the oral microecosystem. In this ecosystem all microorganisms might play some beneficial role not yet revealed. Therefore, broad spectrum antimicrobials in the treatment of oral diseases should be used with caution.

The question of the localization of the microorganisms also arises in case of the mouth, which contains several distinct biotopes. Most periodontal pathogens colonize several niches within the mouth (e.g. sub- and supragingival plaque, saliva, the tongue and the other mucosal surfaces) and the oral soft tissues are considered an important reservoir of periodontal pathogens for colonization or reinfection of the subgingival sites (Asikainen *et al*., 1991; Ximénez-Fyvie *et al*., 2000b; Tanner *et al*., 2002). At the same time, colonization of lactic acid

bacteria varied in different habitats within the mouth. Lactobacilli, although frequently isolated from saliva, were seldom found from subgingival sites, whereas streptococci were frequent in both habitats. Mager *et al*. (2003) showed that the microbiota of saliva resembled that of the dorsum and lateral surfaces of the tongue, and lactobacilli could thus mainly be expected to control the growth of putative periodontal pathogens present on the tongue and in saliva, consequently diminishing the colonization of subgingival sites by these periodontal pathogens. Streptococci, on the other hand, could be the major guardians aiding in maintaining the balance of subgingival microecology.

In summary, our findings indicate that oral lactic acid bacteria may play an important role in the suppression of periodontal pathogens and maintenance of the microecological balance in the oral cavity.

20. Lactobacilli as a perspective tool for treatment of chronic periodontitis

During recent decades lactobacilli have gained importance as probiotics in the treatment of various human diseases. One of the proposed mechanisms of the action of probiotics (e.g. lactobacilli) is their ability to improve colonization resistance against pathogens. As shown in the present study as well as in previous studies, the metabolic end products (such as organic acids) produced by probiotics render low ecological pH which can interfere with the growth of surrounding microorganisms (Sookkhee *et al*., 2001; Annuk *et al*., 2003). In addition, probiotics compete with pathogens for binding sites and available nutrients, and produce antimicrobial substances (Arihara *et al*., 1996; Saito, 2004).

Although chronic periodontitis has a multifactorial etiology, the success of its therapy is dependent on the reduction of periodontal pathogens. In the present study, we chose the approach using lactobacilli as antibody producers against *P. gingivalis* and showed that *Lactobacillus* represents a good candidate microorganism to deliver single-chain antibodies against periodontal pathogens. Single-chain antibodies, which compromise only the binding domain of the immunoglobulins, are easier to produce in bacteria as compared with complete immunoglobulins which are complex molecules (Seegers, 2002). We expressed the functional single-chain fragment scFv derived from the monoclonal antibody 61BG1.3, which is directed against the arginine-specific protease of *P. gingivalis* (RgpA) on the surface of lactobacilli. RgpA protease is capable of mediating the binding of *P. gingivalis* to erythrocyte surface and host macromolecules. The monoclonal antibody 61BG1.3, directed against RgpA, has previously been reported to confer protection against recolonization with *P. gingivalis* in humans (Booth *et al.*, 1996). The biological activity of the recombinant lactobacilli expressing anti-RgpA antibodies was confirmed *in*

vitro; however, animal models will be necessary to show their effectiveness *in vivo* (Genco *et al*., 1998). Transformed lactobacilli expressing functional single-chain antibodies against *S. mutans* have previously been shown to protect rats against dental caries (Krüger *et al*., 2002). Our model could potentially provide at least twofold as high protection against *P. gingivalis*. Firstly, *P. gingivalis*, bound to the surface of transformed lactobacilli, might be effectively killed by the high local concentration of the antimicrobial substances produced by the latter. Secondly, the blockage of *P. gingivalis* cells by antibody carrying lactobacilli could prevent the strong adhesion of the relatively big aggregated particles, e.g. *P. gingivalis* cells, to the mucosa of the gingival crevices. However, according to the data of the present study, lactobacilli are not common inhabitants of the gingival crevice and, therefore, lactobacilli expressing antibodies could potentially interfere with *P. gingivalis* that inhabits oral soft tissues (e.g. the tongue), supragingival plaque and saliva (Petit *et al*., 1994; Ximénez-Fyvie *et al*., 2000b; Mager *et al*., 2003). Alternatively, the recombinant lactobacilli that are able to colonize subgingival sites could be applied directly into the periodontal pocket. Recently, it has been shown that the intra-oral translocation of periodontal pathogens may jeopardise the outcome of periodontal therapy (Quirynen *et al*., 2001) and, therefore, application of recombinant lactobacilli with anti- *P. gingivalis* activity may serve as a useful adjunct diminishing of the spread of periodontal pathogens and of the reinfection of subgingival sites following periodontal treatment.

The limitation of the present plasmid based system is its instability and content of antibiotic resistance markers, however, the development of a chromosomal integrated system for the expression of scFv is going on. The chromosomally integrated system is stable and lacks antibiotic resistance markers. In addition, the chromosomal system may, at least theoretically, allow the expression of antibodies directed against different pathogens, possibly leading to a more synergistic therapeutic effect.

As *in vitro* studies serve as a useful first step in the selection of novel probiotic strains, we used various *in vitro* tests to characterise oral *Lactobacillus* strains of healthy human origin for probiotic properties such as antimicrobial activity against opportunistic pathogens, tolerance of low oral and gastric pH and intestinal bile content, and the absence of transferable antibiotic resistance genes. We found that several strains possessed high antimicrobial activity as well as high tolerance of environmental stress, which make them suitable for using as probiotic candidates. These properties are better expressed in facultatively heterofermentative *L. plantarum*, *L. paracasei* and *L. rhamnosus*, and homofermentative *L. salivarius*. At the same time, the antibiotic susceptibility pattern of strains of *L. plantarum* differed from the natural resistance pattern of lactobacilli, in that they were resistant to tetracyclines. Plasmid-encoded tetracycline resistance has been reported in lactobacilli (Gevers *et al*., 2003) and therefore, the absence of transferable resistance genes in these strains should be confirmed before they can be used as probiotics. In their absence, moderate

natural resistance could be favorable when used in antibiotic/probiotic combination therapies. Based on above described testing, we selected several strains that would be suitable to use as first generation probiotics and as carriers in the development of second generation probiotics. Further research is needed to confirm their clinical effectiveness.

CONCLUSIONS

1. The subgingival microflora of chronic periodontitis patients, unlike that of periodontally healthy subjects, is significantly imbalanced: total subgingival bacterial count in chronic periodontitis patients is much higher than in healthy individuals, a combination of two or three acknowledged periodontal pathogens (*P. gingivalis*, *P. intermedia/nigrescence*, *A. actinomycetemcomitans*) is frequently present, and the prevalent microorganisms appear to be anaerobic by the respiration type and gram-negative by the structure of cell wall. These changes take place at the expense of gram-positive lactic acid bacteria (*S. mutans*- and *S. mitis*-groups of streptococci and/or aerobic coryneforms) characteristic of periodontal health.

2. Although subgingival lactobacilli are missing in chronic periodontitis patients and are scarce in healthy subjects, the prevalence of salivary lactobacilli is equally high in both periodontitis patients and healthy subjects of different ages. Lactobacilli counts in saliva are not dependent on the periodontal status although, as compared with adults, they are lower in schoolchildren.

3. Regardless of the similarity of total counts of salivary lactobacilli, the species composition of the lactoflora in chronic periodontitis patients differs from that in periodontally healthy subjects in the significantly lower occurrence of homofermentative lactobacilli, particularly *L. gasseri*. The inverse association of homofermentative lactobacilli with periodontal pathogens and with the clinical parameters of periodontitis may indicate their periodontal health promoting ability.

4. Oral lactobacilli express significant antimicrobial activity against the acknowledged periodontal pathogens *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*. Several species are antimicrobially effective while addressed to different pathogens: *L. gasseri* is more active against anaerobes, while another frequently found species in periodontally healthy subjects, *L. fermentum*, is more active against microaerophiles. Moreover, another oral lactic acid microorganism, *S. mutans*, that is usually caries-associated, also expresses significant inhibitory activity against periodontal pathogens.

5. Several strains of oral lactobacilli, belonging to different species, possess high antimicrobial activity as well as high tolerance of environmental stress, indicating that they have the potentiality to be used as probiotics. The most

promising strains belong to facultatively heterofermentative *L. plantarum*, *L. paracasei* and *L. rhamnosus*, and homofermentative *L. salivarius*.

6. A novel second generation probiotic, *L. paracasei* expressing biologically active single-chain antibodies against the arginine-specific protease of the periodontal pathogen *P. gingivalis* (RgpA) on its surface, has been developed. The construct expresses the properties necessary for prevention of adhesion of *P. gingivalis* to host tissues: binding capacity to the RgpA antigen, ability to aggregate *P. gingivalis* and antimicrobial activity against *P. gingivalis in vitro*. Animal trials are necessary to confirm its clinical effectiveness in chronic periodontitis.

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In the etiopathogenesis of chronic periodontitis, several disturbances in the microecological balance could play an important role: on one hand, colonization with several putative periodontal pathogens and, on the other hand, the absence and/or reduced number of microorganisms antagonistic to periodontal pathogens, which may lead to the development of chronic periodontitis. Homofermentative lactobacilli, particularly *L. gasseri*, may serve as indicators of periodontal health. Oral lactobacilli have the potentiality to be used as first or second generation probiotics in the prevention and treatment of periodontitis.

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SUMMARY IN ESTONIAN

SUUÕÕNE LAKTOFLOORA KROONILISE PARODONTIIDI JA TERVE PARODONDI KORRAL

Krooniline parodontiit on hammast ümbritsevate kudede ehk parodondi põletik, mille tähtsamaks tekkepõhjuseks peetakse peamiselt hambakatus paiknevaid mikroorganisme. Produtseerides proteolüütilisi ensüüme ja toksilisi ühendeid, on mikroorganismid võimelised otseselt kahjustama hammast ümbritsevaid kudesid ning vallandama põletikulise vastuse igemetes. Seejuures vabanevad mitmesugused põletikumediaatorid, mis võivad põhjustada nii pehmete kudede kui ka luukoe hävimise ja igemetaskute moodustumise.

Suu on koloniseeritud paljude erinevate mikroorganismidega, mis ühelt poolt erinevate sünergistlike ja antagonistlike mehhanismide kaudu tagavad mikroobiökoloogilise tasakaalu terves suus, kuid teiselt poolt on tingimuste muutumisel võimelised põhjustama haigust. Kroonilise parodontiidi tekitajad on peamiselt erinevad gram-negatiivsed mikroorganismid (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actionmycetemcomitans*) ja spiroheedid, mille kasvu soodustab eelkõige igemealuse keskkonna aluseliseks muutumine ja igemevaovedeliku sekretsiooni suurenemine põletiku käigus. Parodontiidi tekitajaid on leitud vähesel määral ka tervetel isikutel, mis näitab, et tüvede haigust tekitav võime võib olla erinev ja/või on tervetel ebasobivad tingimused patogeenide kasvuks. Selliseid ebasobivaid tingimusi saavad luua happeid tootvad mikroorganismid nagu streptokokid ja laktobatsillid, mis oma elutegevuse käigus on võimelised langetama keskkonna pH-d. Sellele olulisele aspektile ei ole seni küllaldast tähelepanu pööratud. Kuigi streptokokke, eeskätt *Streptococcus sanguis*'t, on seostatud terve parodondiga, on laktobatsillide võimalik osa terve parodondi tagamisel jäänud seni ebaselgeks. Pigem on suu laktobatsille seostatud hambakaariese arenguga.

Mujal inimorganismis, nagu seedetraktis ja suguteedes, on laktobatsillid olulised mikroobiökoloogilise tasakaalu tagajad ning on seetõttu leidnud kasutust probiootikumidena. Probiootikum on inimeselt pärinev elus mittepatogeenne mikroob, mille manustamine on tervisele kasulik. Intensiivse uurimistöö käigus on selgunud probiootikumide mitmesugused toimemehhanismid ja kasutusvõimalused. Näiteks on leitud, et *L. rhamnosus* GG sisaldava piima joomine ja *S. mutans*'i vastaseid antikehasid tootvate geneetiliselt muundatud laktobatsillide manustamine vähendab riski haigestuda hambakaariesse. Need uuringud näitavad, et laktobatsille on võimalik kasutada probiootikumidena haiguste ennetamisel ka suus, kuid andmed puuduvad suuõõne laktobatsillide probiootiliste omaduste ja nende kasutamise võimaluste kohta kroonilise parodontiidi ennetamisel ja ravis.

Uurimistöö eesmärgid ja ülesanded

Uurimuse üldiseks eesmärgiks oli hinnata suuõõne laktofloora tähtsust terve parodondi tagamisel ja edendamisel.

Uurimustöö ülesanded:

1. Võrrelda kvalitatiivselt ja kvantitatiivselt kroonilise parodontiidiga ja terve parodondiga isikute igemealust mikrofloorat, et välja selgitada erinevused happeid tootvate mikroorganismide osas.
2. Hinnata igemealuste ja sülje laktobatsillide olemasolu ja hulka parodontiidiga haigetel ja erineva vanusega tervetel isikutel.
3. Võrrelda suuõõne laktobatsillide liigilist koostist parodontiidiga haigetel ja tervetel isikutel.
4. Määrata suuõõne laktobatsillide antimikroobne aktiivsus kroonilise parodontiidi tekitajate suhtes.
5. Iseloomustada suuõõne laktobatsillide omadusi *in vitro* nende võimalikuks kasutamiseks probiootikumidena.
6. Töötada välja teise põlvkonna probiootikum võimalikuks kasutamiseks kroonilise parodontiidi ennetamisel ja ravis.

Uuritav materjal ja meetodid

Uuriti 26 kroonilist parodontiiti põdevat haiget (keskm. vanus 47 a.) ja 15 terve parodondiga (keskm. vanus 37 a.) täiskasvanut ning 96 last (keskm. vanus 12 a.). Haiged olid suunatud TÜ stomatoloogia kliiniku näo- ja lõualuudekirurgia osakonda diagnoosi määramiseks ja raviks. Uuringusse kaasati haiged, kellel kliiniliselt (igemetaskud sügavusega ≤ 5 mm) ja röntenoloogiliselt oli sedastatav parodondi destruktsioon ning kes uuringule eelnenud kuue kuu jooksul ei olnud tarvitanud antibiootikume. Terve parodondiga täiskasvanute rühma moodustasid isikud, kellel puudusid parodondidestruktsioonile iseloomulikud kliinilised ja röngenoloogilised tunnused ning kes ei olnud tarvitanud antibiootikume. Uuritavatel registreeriti kliinilised näitajad: hambakatu ladestused, igemete põletik, mäda esinemine igemetaskutes, igemevao/-tasku sügavus ning hambakaaries. Kõigil parodontiidiga haigetel ja tervetel täiskasvanutel koguti analüüsid 2 igemevaost/-taskust loputusmeetodil ning tehti igemealuse mikrofloora kvantitatiivne ja kvalitatiivne mikrobioloogiline analüüs. Sülje laktobatsillid määrati 20 haigel ja 15 tervel täiskasvanul ning 96 lapsel, kasutades poolkvantitatiivseid Dentocult®LB teste.

Igemealused ja sülje laktobatsillid (kokku 238 tüve) isoleeriti, samastati ja määrati nende grupiline kuuluvus, kasutades biokeemilisi meetodeid; 115 tüve lõplik samastamine toimus molekulaarsete meetoditega (ARDRA, sekveneerimine). Järgnevalt määrati 115 tüve (48 haigetelt ja 67 tervetelt) anti-

mikroobne aktiivsus suuõõne patogeenide *A. actinomycetemcomitans*, *S. mutans* ja *C. albicans* suhtes kahekihilisel agarsöötmel, neist 63 tüve (21 haigetelt ja 42 tervetelt) antimikroobne aktiivsus lisaks veel *P. gingivalis* ja *P. intermedia* suhtes joonkülvi meetodil. Probiootiliste laktobatsillide skriinimiseks valiti 67 tervetelt isikutelt pärinevat tüve, millel lisaks antimikroobsele aktiivsusele määrati vastupanu keskkonna stressile. Määrati tüvede 4 tunni elulemus ja 24 tunni kasv happelises keskkonnas (pH 3,5–1,5) ning 24 tunni kasv sapi manulusel (sapi kontsentratsioonid 0,08–5 %). Antimikroobsete omaduste, happe- ja sapitaluvuse alusel valiti välja 22 parimate omadustega tüve, millel määrati E-test meetodil antibiogramm 13 antibiootikumi suhtes.

Teise põlvkonna probiootikumi väljatöötamiseks viidi *P. gingivalis*'e arginiin-spetsiifilise proteaasi (RgpA) vastaste üheahelaliste antikehade (scFv) sünteesi kodeeriv geen plasmiidsesse ekspressioonivektorisse, mis omakorda sisestati *L. paracasei*'sse. Antikeha ekspressiooni rekombinantse *L. paracasei* pinnal hinnati FACS meetodil ja bioloogilist aktiivsust ELISA ja aglutinatsiooni meetodil. Lisaks määrati rekombinantsete laktobatsillide antimikroobne aktiivsus joonkülvi meetodil.

Uurimistöö tulemused ja järeldused

1. Erinevalt terve parodondiga isikutest esinevad haigete igemealuses mikroflooras olulised tasakaalu nihked: kõrgem mikroobide üldhulk, kahe või kolme tuntud parodntiidi tekitaja (*P. gingivalis*, *P. intermedia/ nigrescens*, *A. actinomycetemcomitans*) sage koosesinemine ning hingamistüübilt anaeroobsete ja rakuseina ehituselt gram-negatiivsete mikroobide ülekaal. Antud muutused toimuvad tervele parodondile iseloomulike gram-positiivsete piimhappe-bakterite, *S.mutans*- ja *S. mitis*- grupi streptokokkide ja/või aeroobsete korüüneformsete bakterite arvel.

2. Kuigi igemealused laktobatsillid puuduvad kroonilise parodontiidiga haigetel ja neid leidub harva tervetel, on laktobatsillide esinemissagedus kõrge süljes – seda nii lastel kui ka täiskasvanutel. Laktobatsillide hulk süljes ei sõltu parodondi seisundist, kuid võrreldes täiskasvanutega on nende hulk madalam lastel.

3. Hoolimata laktobatsillide sarnasest hulgast süljes erineb kroonilise parodontiidiga haigete laktofloora liigiline koostis tervete omast homofermentatiivsete laktobatsillide, eeskätt *L. gasseri,* madalama esinemissageduse poolest. Vastupidiste seoste ilmnemine homofermentatiivsete laktobatsillide ja parodondi patogeenide olemasolu ning parodontiidi kliiniliste näitajate vahel võib osutada selle laktobatsillide grupi võimele tagada terve parodont.

4. Suuõõne laktobatsillidel esineb märkimisväärne antimikroobne aktiivsus tuntud parodontiidi tekitajate *P. gingivalis'*e, *P. intermedia* ja *A. actinomycetemcomitans'*i suhtes. Antimikroobsed omadused on paljudel laktobatsilli liikidel, samas on need suunatud erinevatele patogeenidele: *L. gasseri* on aktiivsem anaeroobide ja *L. fermentum*, teine tervetel sageli esinev liik, mikroaerofiilide suhtes. Lisaks laktobatsillidele on parodontiidi tekitajate kasvu võimeline pärssima ka kariogeenne piimhappe-bakter *S. mutans*.

5. Paljudel suuõõne laktobatsillidel on hea antimikroobne aktiivsus ja kõrge vastupanu kasvukeskkonna stressile, mis näitab nende potentsiaali võimalikuks kasutamiseks probiootikumidena. Parimad tüved kuuluvad heterofermentatiivsete *L. plantarum*, *L. paracasei* ja *L. rhamnosus* ning homofermentatiivsete *L. salivarius* hulka.

6. Välja on töötatud uudne teise põlvkonna probiootikum – *L. paracasei*, mis ekspresseerib oma pinnal üheahelalisi antikehasid parodontiidi tekitaja *P. gingivalis*'e arginiin-spetsiifilise proteaasi vastu. Probiootikumil on olemas omadused, mis on vajalikud *P. gingivalis*'e adhesiooni takistamiseks makroorganismi kudedele: võime seostuda *in vitro* RgpA antigeeniga, aglutineerida ja inhibeerida *P. gingivalis*'t. Probiootikumi kliinilise toime hindamiseks kroonilise parodontiidi ravis on vajalikud uuringud loommudelil.

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Parodontiidi kujunemisel on tõenäoliselt olulised mitmed suuõõne mikroobiökoloogilise tasakaalu häired: ühelt poolt igemealuse mikrofloora koloniseerumine mitme potentsiaalse parodondi patogeeniga, teiselt poolt parodondi patogeenide suhtes antagonistlike mikroobide puudumine ja/või nende vähenenud hulk. Homofermentatiivsed laktobatsillid, eeskätt *L. gasseri*, võivad toimida terve parodondi indikaatoritena. Suu laktobatsillidel on potentsiaali kasutamiseks nii esimese kui teise põlvkonna probiootikumidena kroonilise parodontiidi ennetamisel ja ravis.

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CHARACTERIZATION OF ORAL LACTOBACILLI AS POTENTIAL PROBIOTICS

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ABSTRACT

Our aim was to characterize oral lactobacilli for their antimicrobial properties, survival in various environmental conditions and antibiotic susceptibility reflecting their potential use as probiotics. ***Material and methods***. Sixty-seven salivary and subgingival lactobacilli of periodontally healthy humans were identified using rapid amplified ribosomal DNA restriction analysis and partial sequencing of the 16S-rDNA fragment. Their antimicrobial activity against five oral pathogens, acid and bile tolerance and susceptibility to 13 antibiotics were tested. ***Results***. Ten species were identified. The majority of strains suppressed growth of *Streptococcus mutans*, *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Prevotella intermedia*, but none inhibited *Candida albicans*. The strongest antimicrobial activity was associated with facultatively heterofermentative lactobacilli and homofermentative *L. salivarius*. Sixty-five of 67 strains survived for 4 h at pH 3.0 but only 28 were able to grow at this pH. Survival following 4 h of incubation at pH 2.5 was observed for 25 of 31 strains but none of the strains grew at this pH. Half of the strains were able to grow at bile concentration of 5% (v/v). ***Conclusions***. Strains of *L. plantarum*, *L. paracasei*, *L. salivarius* and *L. rhamnosus* showed both good antimicrobial activity and good tolerance of environmental conditions that make them suitable for using as probiotic candidates. At the same time, the strains of *L. plantarum* differ from the natural resistance pattern of lactobacilli and therefore, the absence of transferable resistance genes in these strains should be confirmed.

Key words: oral lactobacilli, probiotic, antimicrobial activity, acid tolerance, bile tolerance, antibiotic susceptibility

INTRODUCTION

Lactobacilli, the acidophilic and aciduric gram-positive bacteria of the genus *Lactobacillus*, belong to the indigenous microflora of humans and colonize various parts of the body (Axelsson, 2004). Lactobacilli are known to play an important role in the maintenance of human health by stimulating the natural immunity and contributing to the balance of microflora, mainly through competitive exclusion and antimicrobial activity against pathogenic bacteria (McFarland, 2000; Perdigon *et al*., 2001, Mikelsaar *et al*., 2004). Several species of obligately homofermentative (OHOL), facultatively (FHEL) and obligately heterofermentative (OHEL) lactobacilli have been found in the oral cavity, with *Lactobacillus gasseri*, *L. salivarius*, *L. plantarum*, *L. rhamnosus* and *L. fermentum*, being the most prevalent (Ahrne *et al*., 1998; Colloca *et al*., 2000; Kõll-Klais *et al*., 2005).

Lactobacilli are widely used for the manufacturing of fermented foodstuffs and as such, have been consumed for centuries. During recent decades lactobacilli have gained importance as probiotics, "*live microorganisms which when administered confer a health benefit on the host*" (FAO / WHO, 2002). These beneficial effects range from reducing gastrointestinal disorders to protecting against colon cancer (Isolauri *et al*., 2002), but the underlying mechanisms that cause these effects are still poorly understood. Few studies are available on the role and effects of probiotics in the mouth (Meurman, 2005). Näse *et al*. (2001) showed that long-term consumption of milk containing *L. rhamnosus* GG caused a significant reduction in caries risk in day-care children. Short-time consumption of *L. rhamnosus* GG and *L. rhamnosus* LC 705 containing cheese did also, to some extent, decrease the number of mutans streptococci (Ahola *et al*., 2002). In the field of oral immunology, administration of transformed lactobacilli, expressing single-chain antibody fragments against *Streptococcus mutans*, has been shown to protect rats against the development of dental caries (Krüger *et al.*, 2002). Furthermore, we have expressed functional single chain antibody fragments against *Porphyromonas gingivalis* in *Lactobacillus paracasei* to combat periodontal diseases (Marcotte *et al*., 2006). Thus, these studies point out the possibility of using lactobacilli as probiotics not only against gastrointestinal disorders but also against oral diseases. However, little is known about the probiotic properties of oral lactobacilli.

Several requirements have been proposed for novel probiotic strains. Isolates from healthy humans are advised (Saarela *et al*., 2000). Strains should be able to colonize the site in which their beneficial action is expected and survive passage through the acidic gastric environment and tolerate the effects of bile when used in the gastrointestinal tract (Saarela *et al*., 2000). In addition, good antimicrobial properties of probiotic strains could give additional positive effect in eradicating or inhibiting the target bacteria. At the same time significant fermentation type-, species- and strain-specific variability in anti-

microbial activity as well as in acid and bile tolerance of lactobacilli has been observed (Jacobsen *et al*., 1999; Sookkhee *et al*., 2001; Annuk *et al*., 2003) and therefore, several strains from various fermentation types and species should be tested to choose the best ones. Furthermore, there is growing concern about the development of antibiotic resistance in pathogenic microorganisms. The spread of antibiotic-resistant genes among bacterial species may occur through lateral gene transfer (Duncan, 2003) and therefore, the resistance pattern of the probiotic strains would be useful to know to avoid inducing strains that carry transferable resistance genes.

The aim of the present study was to characterize oral lactobacilli for their antimicrobial properties, survival in various environmental conditions and antibiotic susceptibility reflecting their potential use as probiotics.

MATERIAL AND METHODS

Sampling, isolation and identification of lactobacilli

Sixty-seven oral lactobacilli strains used in this study (Table 1) were isolated from saliva and subgingival samples of 11 periodontally healthy humans (mean age 36.2 ± 10.5 years) participating in a prospective study of oral microflora in periodontitis and periodontal health. The study design, selection of patients, isolation and identification of lactobacilli have been thoroughly described elsewhere (Kõll-Klais *et al*., 2005). Briefly, salivary lactobacilli (60 strains) were obtained by using Dentocult®LB dip-slide (Orion Diagnostica, Espoo, Finland) method (Birkhed *et al*., 1981) and subgingival lactobacilli (7 strains) were obtained by plating the gingival crevice lavage samples (Boström *et al*., 1998) onto MRS agar. Provisional identification was based on the morphological, physiological and biochemical properties (Kandler and Weiss, 1986), the species were identified using rapid amplified ribosomal DNA restriction analysis (ARDRA) (Ventura *et al*., 2000). Partial sequencing of the 16S-rDNA fragment was performed for strains with uncertain identity.

Testing of antimicrobial activity

Target bacterial strains used for antimicrobial activity testing were *Streptococcus mutans* NG8 (wild type), *Actinobacillus actinomycetemcomitans* 31-2-1A (wild type), *Porphyromonas gingivalis* ATCC 49 417, *Prevotella intermedia* ATCC 25 611 and *Candida albicans* 048 (wild type).

Antimicrobial activity testing of lactobacilli against microaerophilic target bacteria *S. mutans* and *A. actinomycetemcomitans* (67 lactobacilli strains were tested) and anaerobic target bacteria *P. gingivalis* and *P. intermedia* (42 lactobacilli strains were tested) has been described elsewhere (Kõll-Klais *et al*., 2005).

The antimicrobial activity of lactobacilli against *C. albicans* was assessed by the deferred antagonism method (Morency *et al*., 2001). The medium used as bottom agar (1.4%) was MRS agar without tri-ammonium-citrate and sodium-acetate (pH 7.1) (Annuk *et al*., 2003). The medium used as top agar (0.7%) was Sabouraud-2% dextrose (Merck). Lactobacilli were stab-inoculated on the surface of the bottom agar and incubated anaerobically (BBL®*GasPakPlus*™, BBL Microbiology Systems, Cockeysville, MD) for 24 h at 37ºC to develop visible macrocolonies. A maximum of four *Lactobacillus* strains were grown on one agar plate. The target bacteria were precultivated in Sabouraud-2% dextrose (Merck) broth and suspensions of cells were adjusted to a predetermined optical density (OD 0.10 at 600 nm) to yield confluent growth in the top agar. Thereafter, the melted (and cooled to 42°C) top agar was seeded with the precultivated target bacterial suspension and poured over the macrocolonies of lactobacilli. The plates were incubated under microaerobic conditions (BBL®*CampyPakPlus*™, BBL Microbiology Systems, Cockeysville, MD) at 37ºC for 24 h to yield inhibitory zones. The tests were performed in duplicate, and the results were reported as the mean width of two inhibition zones measured from the edge of the colony of *Lactobacillus* strain to the margin of the inhibition zone.

Testing of acid tolerance

Survival testing

The effect of low pH on the survival of lactobacilli was examined in flat-bottom microwell plates (Costar® 96 Well Cell Culture Cluster, Myriad Industries, San Diego, CA) with MRS broth (Merck) adjusted to pH 3.5 to 1.5 with 6N HCl, and a non-adjusted MRS broth (pH 5.6) as control. Each 180-µl volume of pH adjusted or non-adjusted MRS broth were inoculated with the 20-µl of overnight culture of lactobacilli (containing about 10^6 to 10^7 CFU/ml) and incubated aerobically at 37ºC for 4 h. The number of surviving cells was determined by plating 100-µl of tenfold serially diluted sample onto the MRS agar (Jacobsen *et al*., 1999). In total, 67 strains were tested at pH 3.5 and 3.0, and 31 strains were additionally tested at pH 2.5, 2.0 and 1.5.

Growth testing

In parallel, growth at pH 3.5 to 1.5 was measured as changes in optical density (OD) in 180-µl volume of pH-adjusted and non-adjusted (as control) MRS broth, inoculated with 20-µl of overnight culture of lactobacilli (containing about 10^6 to 10^7 CFU/ml), at 630 nm (OD_{630}) following 0, 3, 6, 9, 12 and 24 h of incubation at 37ºC in aerobic conditions (Jacobsen *et al*., 1999). In total, 67 strains were tested at pH 3.5 and 3.0, and 23 strains were additionally tested at pH 2.5, 2.0 and 1.5.

Testing of bile tolerance

The effect of bile salts on the growth of lactobacilli (67 strains) was examined by adding human bile to MRS broth to a final concentration of 0.08, 0.16, 0.3, 0.6, 1.25, 2.5 and 5.0 % (v/v). A 180-µl volume of bile adjusted and non-adjusted (as control) MRS broth were inoculated with the 20-µl of overnight culture of lactobacilli (containing about 10^6 to 10^7 CFU/ml) and incubated aerobically at 37ºC for 24 h. Changes in OD at 630 nm (OD_{630}) were measured following 0, 3, 6, 9, 12 and 24 h of incubation (Jacobsen *et al*., 1999).

Selection of best lactobacilli strains

Each particular *Lactobacillus* strain was scored according to the results of antimicrobial activity, acid and bile tolerance with maximum value of 19. Antimicrobial activity of a strain determined by the deferred antagonism method (*S. mutans*, *A. actinomycetemcomitans* and *C. albicans*) was scored as follows: 0, inhibition less than 1 mm; 1, equal to or above 1 and less than 2 mm of inhibition; 2, between 2 and 5 mm of inhibition; 3, 5 mm of inhibition and above; and by the streak line procedure (*P. gingivalis* and *P. intrmedia*) as follows: 0, inhibition less than 1 mm; 1, equal to or above 1 and less than 7 mm of inhibition; 2, between 7 and 20 mm of inhibition; 3, 20 mm of inhibition and above. Acid tolerance was scored as follows: 0, survival or growth only at pH higher than 3.5; 1, survival or growth at pH 3.5; 2, survival or growth at pH 3.0 or less. Bile tolerance was scored as follows: 0, growth only at bile concentrations lower than 0.6%; 1, growth at bile concentrations 0.6 or 1.25%; 2, growth at bile concentration 2.5% or higher.

Testing of antibiotic susceptibility

Twenty two strains were tested. Minimum inhibitory concentrations (MICs) of inhibitors of cell wall synthesis (amoxicillin, cefoxitin, cefprozil, cefotaxime, vancomycin), protein synthesis (gentamicin, erythromycin, doxycycline, tetracycline, clindamycin, chloramphenicol) and nucleic acid synthesis (ciprofloxacin, metronidazole) were determined by E-test method. Saline solution for suspending bacteria (McFarland 0.5 turbidity standard), Wilkins-Chalgren (Oxoid) agar plates with 5% horse blood and E-test antibiotic strips (AB Biodisk, Solna, Sweden) were used. After 48 h of incubation at 37ºC in an anaerobic glove chamber (Sheldon Manufacturing, Inc. Shel LAB), the elliptical zones of growth inhibition were examined and the MICs were interpreted as the value on the E-test strip scale where the inhibition zone intersected the edge of the strip. The breakpoints (susceptible/resistant) were determined in accordance with National Committee for Clinical Laboratory Standards (NCCLS) guidelines for gram-positive microorganisms (Jorgensen and Turnidge, 2003) as follows: clindamycin and ciprofloxacin (4 µg/ml); amoxicillin and erythromycin (8 µg/ml); gentamicin, doxycycline and tetracycline (16 µg/ml); cefoxitin, cefprozil, vancomycin, chloramphenicol and metronidazole

(32 μg/ml); and cefotaxime (64 μg/ml). Strains with MICs equal to or higher than breakpoints were considered as resistant.

Statistical methods

Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA) and Excel (Microsoft Corp., Redmond, WA) programs. The following tests were employed: *t*-test and Mann-Whitney rank sum test (comparison of different bacterial groups). The differences were considered significant when P value was < 0.05.

RESULTS

Identification of *Lactobacillus* species

The distribution of different fermentation types of lactobacilli was as follows: 22 in OHOL, 18 in FHEL and 27 in OHEL group (Table 1). All 67 strains were subjected to ARDRA analysis, and of those, 66 isolates were identified by ARDRA as *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. rhamnosus* and *L. fermentum*. Nineteen strains were later reassigned based on results from the 16S-rRNA gene sequencing (first 500 bases of the16S-rRNA gene). The latter included 8 strains of *L. casei* to *L. paracasei* ssp. *paracasei* and 11 strains of *L. fermentum* to *L. oris*. One strain which showed an unknown restriction pattern by ARDRA was identified following sequencing of the 16S-rRNA gene as *L. delbrueckii*.

Table 1. Species and origin of oral lactobacilli characterized for probiotic use.

Lactobacilli	Strains (n) isolated from	
Fermentation type/ species	saliva	subgingival sites
OHOL	19	3
L. acidophilus	2	
L. crispatus	2	
L. delbrueckii	1	
L. gasseri	9	3
L. salivarius	5	
FHEL	17	1
L. paracasei	7	1
L. plantarum	7	
L. rhamnosus	3	
OHEL	24	3
L. fermentum	16	
L. oris	8	3

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.

Antimicrobial activity

The majority of *Lactobacillus* strains suppressed growth of *A. actinomycetemcomitans* (89% of tested strains), *P. gingivalis* (83%), *P. intermedia* (62%) and *S. mutans* (55%), but none inhibited *C. albicans*. The antimicrobial activity was mainly species specific; however, some strain specific differences were observed, particularly among strains of *L. fermentum*, *L. oris* and, *L. gasseri*. The strongest antimicrobial activity was associated with facultatively heterofermentative lactobacilli and homofermentative *L. salivarius* (Table 2). In addition, homofermentative *L. crispatus* and *L. gasseri* had quite high activity against anaerobic *P. gingivalis* and *P. intermedia*, whereas *L. fermentum* (OHEL) inhibited neither of these anaerobic bacteria. Lactobacilli from all fermentation types showed higher antimicrobial activity against *P. gingivalis* than against *P. intermedia* (inhibition zone 18.2 ± 5.5 vs. 6.5 ± 3.8 mm, $P<0.001$, in OHOL group; 22.4 ± 3.4 vs. 10.6 ± 1.4 mm, $P<0.001$, in FHEL group; 7.1 ± 7.5 vs. 0 mm, $P<0.05$, in OHEL group).

The inhibitory activity of subgingival strains (*L. gasseri*, *L. paracasei* and *L. oris*) was comparable to the same species isolated from saliva (Table 2).

Table 2. Antimicrobial activity of oral lactobacilli originating from saliva and subgingival sites, expressed as inhibition zone values (mm).

Lacto-bacilli		Inhibition of target bacteria: zone values (mm) mean ± SD				
Origin	Fermentation type/ species	*S. mutans*	*A. actinomy-cetemcomitans*	*P. gingivalis*	*P. intermedia*	*C. albi-cans*
Salivary	OHOL, *n* =	*19*	*19*	*15*	*15*	*19*
	L. acidophilus	0	2.5 ± 0	12.0 ± 0	0	0
	L. crispatus	0	1.0 ± 0.7	26.7 ± 0	9.5 ± 0	0
	L. delbrueckii	1.5 ± 0	4.3 ± 0	11.3 ± 0	0	0
	L. gasseri	0.1 ± 0.2	1.5 ± 0.9	17.1 ± 4.0	6.7 ± 2.4	0
	L. salivarius	2.7 ± 2.1	4.2 ± 0.8	24.4 ± 4.2	11.2 ± 1.9	0
	FHEL, *n* =	*17*	*17*	*9*	*9*	*17*
	L. paracasei	2.2 ± 1.5	3.7 ± 1.2	24.0 ± 2.1	12.0 ± 1.9	0
	L. plantarum	3.0 ± 0.8	6.1 ± 0.7	21.7 ± 5.4	9.6 ± 1.3	0
	L. rhamnosus	2.0 ± 0.5	4.4 ± 1.0	22.1 ± 1.2	11.2 ± 0.3	0
	OHEL, *n* =	*24*	*24*	*12*	*12*	*24*
	L. fermentum	1.3 ± 0.8	3.1 ± 2.4	0	0	0
	L. oris	0.1 ± 0.4	2.4 ± 1.5	12.1 ± 5.7	0	0

Table 2. (Continuation)

Lacto-bacilli		Inhibition of target bacteria: zone values (mm) mean ± SD				
Origin	Fermentation type/ species	*S. mutans*	*A. actinomy-cetemcomitans*	*P. gingivalis*	*P. intermedia*	*C. albi-cans*
Sub-gingival	OHOL, *n* =	*3*	*3*	*3*	*3*	*3*
	L. gasseri	0	1.8 ± 0.4	17.4 ± 5.1	4.5 ± 3.0	0
	FHEL, *n* =	*1*	*1*	*1*	*1*	*1*
	L. paracasei	2.5 ± 0	6.0 ± 0	23.0 ± 0	10.3 ± 0	0
	OHEL, *n* =	*3*	*3*	*2*	*2*	*3*
	L. oris	0	1.1 ± 1.9	7.4 ± 7.9	0	0

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.

Acid tolerance

Acid tolerance by lactobacilli was found to be strain-, species- and fermentation type-specific (Table 3). Nearly all strains (65 of 67) survived for 4 h at pH 3.0 but only 28 were able to grow at this pH. Survival following 4 h of incubation at pH 2.5 was observed for 25 (10 FHEL, 15 OHEL strains) of 31 strains but none of the strains tested grew at this pH. Heterofermentative *L. plantarum* and *L. fermentum* were the most tolerant species. The acid tolerance of subgingival strains was comparable to the same species isolated from saliva.

Table 3. Survival and growth of lactobacilli in an acidic environment, expressed as a percentage (%) of surviving and growing strains.

Lactobacilli			Growth[1] of strains (%) at pH			Survival[2] of strains (%) at pH						
			5.6	3.5	3.0[§]	5.6	3.5	3.0		2.5	2.0	1.5
Origin	Fermen-tation type/ species	No. of strains tested							No. of strains tested			
Sali-vary	OHOL											
	L. acidophilus	*2*	100	0	0	100	100	100				
	L. crispatus	*2*	100	0	0	100	100	100				
	L. delbrueckii	*1*	100	0	0	100	0	0				
	L. gasseri	*9*	100	22	0	100	100	89	*1*	0	0	0
	L. salivarius	*5*	100	80	0	100	100	100	*3*	0	0	0
	FHEL											
	L. paracasei	*7*	100	86	29	100	100	100	*1*	100	0	0
	L. plantarum	*7*	100	100	100	100	100	100	*7*	100	0	0
	L. rhamnosus	*3*	100	100	33	100	100	100	*3*	67	0	0

Table 3. (Continuation)

Lactobacilli			Growth[1] of strains (%) at pH			Survival[2] of strains (%) at pH						
			5.6	3.5	3.0[§]	5.6	3.5	3.0		2.5	2.0	1.5
Origin	Fermen-tation type/ species	No. of strains tested							No. of strains tested			
	OHEL											
	L. fermentum	*16*	100	100	100	100	100	100	*15*	100	0	0
	L. oris	*8*	100	100	25	100	100	100				
Subgin-gival												
	L. gasseri	*3*	100	0	0	100	100	100				
	L. paracasei	*1*	100	100	0	100	100	100	*1*	0	0	0
	L. oris	*3*	100	100	0	100	100	100				

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.
[1]Data presented following incubation for 24 hours: [§], no growth was observed at pH lower than 3.0
[2]Data presented following incubation for 4 hours

Bile tolerance

The tested strains showed relatively high tolerance of bile salts: half of the strains (11 OHOL, 14 FHEL, 8 OHEL) were able to grow at bile concentration of 5% (v/v) following 24 h of incubation (Table 4). The most tolerant species were heterofermentative *L. paracasei* and *L. rhamnosus*, and homofermentative *L. acidophilus*. In Fig. 1 the growth curve of lactobacilli at various concentrations of bile is shown. The bile tolerance of subgingival strains was comparable to the same species isolated from saliva.

Table 4. Growth of lactobacilli at various concentrations of bile following incubation for 24 h, expressed as a percentage (%) of growing strains.

Lactobacilli			Growth of strains (%) at bile concentration (% v/v)						
Origin	Fermentation type/species	No. of strains tested	0.08	0.16	0.3	0.6	1.25	2.5	5
Salivary	OHOL								
	L. acidophilus	*2*	100	100	100	100	100	100	100
	L. crispatus	*2*	100	100	100	100	100	50	0
	L. delbrueckii	*1*	100	100	100	100	100	100	0
	L. gasseri	*9*	100	100	100	89	89	78	56
	L. salivarius	*5*	100	100	100	100	100	100	60
	FHEL								
	L. paracasei	*7*	100	100	100	100	100	100	100
	L. plantarum	*7*	100	100	100	100	100	100	43
	L. rhamnosus	*3*	100	100	100	100	100	100	100
	OHEL								
	L. fermentum	*16*	100	100	100	100	94	19	0
	L. oris	*8*	100	100	100	100	100	100	75
Subgingival									
	L. gasseri	*3*	100	100	100	100	67	33	33
	L. paracasei	*1*	100	100	100	100	100	100	100
	L. oris	*3*	100	100	100	100	100	100	67

OHOL, obligately homofermentative; FHEL, facultatively heterofermentative; OHEL, obligately heterofermentative.

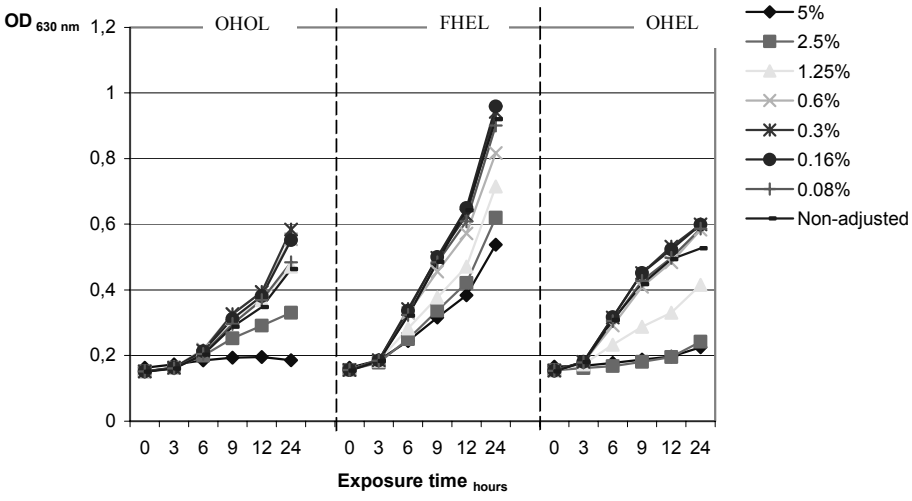

Figure 1. Changes in optical density (OD) during growth of lactobacilli at different concentrations of bile. Data are presented as a mean value for each fermentation group at a particular time point.

Selection of the best lactobacilli strains

Each particular *Lactobacillus* strain was scored according to the results of antimicrobial activity, acid and bile tolerance. Data for 18 salivary and 4 subgingival strains are shown in Table 5. Strains of species of *L. plantarum*, *L. paracasei*, *L. salivarius* and *L. rhamnosus* showed both high antimicrobial activity and good tolerance of low pH and high concentration of bile.

Antibiotic susceptibility

Data regarding susceptibility of 22 *Lactobacillus* strains to 13 antibiotics are presented in Table 6. No resistance was found to amoxicillin, cefprozil, cefotaxime, erythromycin and chloramphenicol. Although most of the strains had low MICs to gentamicin, doxycycline, tetracycline and clindamycin, some resistant strains appeared. One strain of *L. gasseri* was resistant to gentamicin, all 4 *L. plantarum* and 2 *L. oris* strains, to doxycycline and/or tetracycline and all 4 *L. gasseri* strains to clindamycin. All studied lactobacilli were resistant to metronidazole and majority of the strains, belonging to different species, were resistant to cefoxitin, vancomycin and ciprofloxacin. All vancomycin susceptible strains belonged to *L. gasseri,* while cefoxitin and ciprofloxacin susceptible strains belonged to species of *L. gasseri*, *L. salivarius* and *L. paracasei*. When testing for ciprofloxacin (2 *L. salivarius*, 1 *L. paracasei* and 2 *L. rhamnosus* strains), cefotaxime (2 *L. oris*) and cefprozil (2 *L. fermentum*), pinpoint colonies were observed within the inhibition zone of the E-test. Following re-testing of the strains, these colonies were considered as resistant mutants and were included in the MICs.

Table 5. Antimicrobial activity, acid and bile tolerance of 22 selected salivary and subgingival *Lactobacillus* strains.

Lactobacilli			Antimicrobial activity toward					Survi-val[3] at	Growth[4] at		Score[4]
Origin	Strain	Species	Sm[1]	Aa[1]	Pg[2]	Pi[2]	Ca[1]	pH	pH	bile (% v/v)	
Saliva	25-DLB-3A-A	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	27-DLB-1	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	35-DLB-2	*L. plantarum*	+	++	++	+	–	2.5	3.0	2.5	16
	21-DLB-4-B	*L. paracasei*	+	++	++	+	–	3.0[§]	3.0	5	16
	37-DLB-2A	*L. plantarum*	+	++	+	+	–	2.5	3.0	5	15
	27-DLB-2A	*L. salivarius*	++	+	++	+	–	3.0	3.5	2.5	15
	43-DLB-3-A	*L. salivarius*	+	++	++	+	–	3.0	3.5	5	15
	21-DLB-5-B	*L. rhamnosus*	+	++	++	+	–	2.5	3.5	5	15
	35-DLB-5	*L. rhamnosus*	–	++	++	+	–	3.0	3.0	5	15
	21-DLB-6-B	*L. paracasei*	+	+	++	+	–	3.0[§]	3.5	5	14
	37-DLB-1	*L. salivarius*	+	+	++	+	–	3.0	3.5	5	14
	21-DLB-7	*L. rhamnosus*	+	+	++	+	–	2.5	3.5	5	14
	13-DLB-6A	*L. gasseri*	–	+	++	+	–	3.0[§]	3.5	1.25	11
	33-DLB-2	*L. gasseri*	–	+	+	+	–	3.0	3.5	5	11
	13-DLB-4A	*L. fermentum*	+	++	–	–	–	2.5	3.0	1.25	10
	37-DLB-2B	*L. fermentum*	–	++	–	–	–	2.5	3.0	2.5	10
	21-DLB-1B-2	*L. oris*	–	+	+	–	–	3.0[§]	3.0	5	10
	25-DLB-4A-1	*L. oris*	–	++	+	–	–	3.0[§]	3.5	5	10
Sub-gingival	8-2-16A-B	*L. paracasei*	+	++	++	+	–	3.0	3.5	5	15
	8-2-1A	*L. gasseri*	–	–	+	+	–	3.0[§]	5.6	5	9
	37-2-10-A	*L. gasseri*	–	+	++	–	–	3.0[§]	5.6	1.25	9
	8-2-16B	*L. oris*	–	+	+	–	–	3.0[§]	3.5	5	9

[1]Antimicrobial activity: -, no inhibition or less than 2mm; +, between 2 and 5 mm of inhibition; ++, 5 mm of inhibition and above; Sm, *S. mutans*; Aa, *A. actinomycetemcomitans*; Ca, *C. albicans*.
[2]Antimicrobial activity: -, no inhibition or less than 7 mm; +, between 7 and 20 mm of inhibition; ++, 20 mm of inhibition and above; Pg, *P. gingivalis*; Pi, *P. intermedia*.
[3]Data presented following incubation for 4 hours: [§], not determined at pH lower than 3.0.
[4]Data presented following incubation for 24 hours.
[5]Total score for a *Lactobacillus* strain based on the data of antimicrobial activity, acid and bile tolerance (max. value 19).

Table 6. Antibiotic susceptibility of 22 selected salivary and subgingival *Lactobacillus* strains.

Lactobacilli		Antibiotic[1] with the MIC (μg/ml) as follows:												
Strain	Species	AC	FX	FP	CT	VA	GM	EM	DC	TC	CM	CL	CI	MZ
Salivary strains														
25-DLB-3A-A	*L. plantarum*	0.25	≥256	0.5	0.25	≥256	2	0.38	16	24	0.38	3	≥32	≥256
27-DLB-1	*L. plantarum*	0.38	≥256	0.5	0.38	≥256	1.5	0.5	24	24	0.25	4	≥32	≥256
35-DLB-2	*L. plantarum*	0.19	≥256	0.38	0.25	≥256	1.5	0.25	6	24	1.5	4	≥32	≥256
21-DLB-4-B	*L. paracasei*	1.0	≥256	6	6	≥256	4	0.19	0.75	0.75	0.09	3	2	≥256
37-DLB-2A	*L. plantarum*	0.12	≥256	0.38	0.19	≥256	1.0	0.5	16	32	0.75	4	≥32	≥256
27-DLB-2A	*L. salivarius*	0.75	16	1.5	0.75	≥256	12	0.75	1.0	1.5	0.25	2	1.5	≥256
43-DLB-3-A	*L. salivarius*	0.19	1.5	0.75	0.25	≥256	2	0.25	0.25	0.25	0.19	1.5	≥32	≥256
21-DLB-5-B	*L. rhamnosus*	0.5	≥256	6	4	≥256	8	0.38	0.5	1.0	0.38	4	12	≥256
35-DLB-5	*L. rhamnosus*	0.75	≥256	8	6	≥256	12	0.25	0.5	0.5	0.75	3	4	≥256
21-DLB-6-B	*L. paracasei*	0.75	≥256	6	6	≥256	4	0.19	0.75	0.75	0.25	3	6	≥256
37-DLB-1	*L. salivarius*	0.25	6	0.75	0.38	≥256	12	0.38	0.75	1.0	0.19	2	≥32	≥256
21-DLB-7	*L. rhamnosus*	0.75	≥256	8	6	≥256	8	0.25	0.5	0.5	0.5	3	12	≥256
13-DLB-6A	*L. gasseri*	0.38	≥256	3	1.0	1.5	8	0.19	2	3	8	6	≥32	≥256
33-DLB-2	*L. gasseri*	0.5	≥256	1.5	2	1.5	12	0.09	1.5	0.75	8	3	≥32	≥256
13-DLB-4A	*L. fermentum*	0.25	≥256	12	0.5	≥256	1.5	0.12	3	2	0.01	3	≥32	≥256
37-DLB-2B	*L. fermentum*	0.25	128	0.75	0.5	≥256	0.25	0.09	6	6	0.02	3	6	≥256
21-DLB-1B-2	*L. oris*	0.38	≥256	3	1.5	≥256	2	0.19	16	24	0.03	6	≥32	≥256
25-DLB-4A-1	*L. oris*	0.25	≥256	4	1.5	≥256	0.75	0.19	12	16	0.12	4	≥32	≥256
Subgingival strains														
8-2-16A-B	*L. paracasei*	1.0	≥256	8	12	≥256	8	0.25	0.75	0.75	0.25	3	1.0	≥256
8-2-1A	*L. gasseri*	0.19	32	1.5	0.38	1.5	16	0.19	1.5	1.5	48	3	≥32	≥256
37-2-10-A	*L. gasseri*	0.19	4	1.5	0.5	1.5	6	0.12	0.38	0.38	4	2	≥32	≥256
8-2-16B	*L. oris*	0.38	≥256	2	0.38	≥256	0.25	0.12	12	12	0.19	3	≥32	≥256

[1]AC, amoxicillin; FX, cefoxitin; FP, cefprozil; CT, cefotaxime; VA, vancomycin; GM, gentamicin; EM, erythromycin; DC, doxycycline; TC, tetracycline; CM, clindamycin; CL, chloramphenicol; CI, ciprofloxacin; MZ, metronidazole.

DISCUSSION

As *in vitro* studies serve as a useful first step in the selection of potential probiotics, we used various *in vitro* tests to characterise human oral lactobacilli for probiotic properties: antimicrobial activity against opportunistic pathogens, tolerance of low oral and gastric pH and intestinal bile content, and the absence of transferable antibiotic resistance genes.

We found that most of the oral *Lactobacillus* strains showed antimicrobial activity against putative oral pathogens. Yet as shown also for intestinal lactobacilli (Annuk *et al.,* 2003), it was largely fermentation group-, species- and strain-specific. Facultatively heterofermentative lactobacilli (*L. plantarum, L. paracasei*, *L. rhamnosus*) and homofermentative *L. salivarius* expressed the strongest antimicrobial activity, which is consistent with their phylogenetic relatedness, both belonging to the *Lactobacillus casei* - *Pediococcus* group. Good antimicrobial activity of oral *L. paracasei* and *L. rhamnosus* against oral pathogens has previously been shown by Sookkhee *et al.* (2001). In contrary, Testa *et al.* (2003) found no antagonistic interactions between oral lactobacilli (*L. casei*, *L. rhamnosus*, *L. plantarum* and *L. salivarius*) and the anaerobes *P. intermedia* and *Fusobacterium nucleatum.* In the present study, we also tested the ability of oral lactobacilli to inhibit the growth of *C. albicans*, but found no inhibition. Some anticandidal effect of strains of *L. paracasei* and *L. rhamnosus* was found by Sookkhee *et al.* (2001), but no inhibition of *C. albicans* by lactobacilli was seen when Mastromarino *et al.* (2002) characterized vaginal lactobacilli. These conflicting results regarding antimicrobial activity could be due to differences in methodology, but could also be related to microbial factors, like the strain-specific antimicrobial activity of lactobacilli as well as variability in sensitivity of different target strains. Our earlier results have shown that the antimicrobial activity of lactobacilli could be related to the production of organic acids, such as lactic and acetic acid, upon fermentation of glucose with a concomitant decrease in pH (Annuk *et al*., 2003). In addition, some lactobacilli produce hydrogen peroxide and bacteriocins (Mastromarino *et al*., 2002; Annuk *et al*., 2003; Zhu *et al*., 2000).

In order to survive in and colonize the gastrointestinal tract, the bacteria must survive passage through the acidic oral and gastric environment and tolerate the effects of bile produced by the small intestine of humans (Ouwehand *et al*., 1999). Probiotic strains that are able to survive and grow at the physiological levels of bile and low pH *in vitro* are more likely to survive in the intestinal transit. In the present study, both the pH and bile tolerance was higher among heterofermentative strains, yet as seen for the antimicrobial activity, the properties were largely strain- and species-specific. The lowest pH that was resisted by lactobacilli following 4 hours of incubation was 2.5, with *L. fermentum* and *L. plantarum* being most stable species. But even the most resistant strains were not able to replicate at this pH, confirming the results by Jacobsen *et al.* (1999). In another study, different *Lactobacillus* strains (duck

origin) were shown to survive an incubation period of 4 h at pH 2.0, with *L. salivarius* TMW 1.992 and *Lactobacillus animalis* TMW 1.972 surviving after an incubation at pH 1.0 for 1h (Ehrmann *et al*., 2002). Fernandez *et al*. (2003) demonstrated that homofermentative *L. acidophilus* and *L. gasseri* were able to survive pH 2.0 during 90 min. These results indicate that both pH and time are essential factors that determine the survival of strains during passage through the acidic environment in the stomach. The pH of the stomach may fall as low as 1.0, but when food comes into the stomach, pH may rise to levels of 3.0 to 4.0 level due to the buffering capacity of proteins. In resting dental plaque pH has been reported to be around 6.5 (ranging between 5.6 and 7.0), with a drop down to 4.5 and 4.0 following a sucrose rinse (Nyvad and Fejerskov, 1994). Most of the studied strains resisted incubation at pH 3.0 for 4 h and tolerated well different concentrations of bile salts, which makes them good candidates for use as probiotics.

An important requirement for probiotic strains is that they should not carry transferable antibiotic resistance genes. The spread of such genes among bacterial species through lateral gene transfer may contribute to dissemination of resistance to antibiotics used for therapy (Saarela *et al*., 2000; European Commission, 2001; Duncan, 2003). Based on the results of antimicrobial activity, acid and bile tolerance we selected 22 (18 salivary and 4 subgingival) most promising lactobacilli for use as probiotics and screened them for a group of 13 antibiotics. Inhibitors of cell wall, protein and nucleic acid synthesis were included. We found that oral lactobacilli, as we have also observed for intestinal lactobacilli (Mändar *et al*., 2001), did not display uniform susceptibility to antibiotics. Although most of the strains were sensitive to a number of clinically effective antibiotics, high level of resistance to cefoxitin, vancomycin, ciprofloxacin and metronidazole was found. Similar results have previously been reported (Hamilton-Miller and Shah, 1998; Mändar *et al*., 2001; Danielsen and Wind, 2003) and could be interpreted as high natural resistance to these antibiotics. Metronidazole resistance, being considered as *Lactobacillus* genus specific natural resistance, is linked to the absence of hydrogenase activity in lactobacilli (Church *et al*., 1996). At the same time, susceptibility to vancomycin has been found to be *Lactobacillus* species related, with all heterofermentative lactobacilli being vancomycin resistant and susceptible ones belonging to OHOL group (Felten *et al*., 1999; Mändar *et al*., 2001; Danielsen and Wind, 2003; Delgado *et al*., 2005; Lenzner *et al*., 1980). Natural vancomycin resistance is related to the production of cell wall peptidoglycan precursors terminating in D-alanine-D-lactate, to which vancomycin does not bind (Handwerger *et al*., 1994). Our study results, where all heterofermentative lactobacilli and *L. salivarius* were resistant to vancomycin, and strains of *L. gasseri* were susceptible, are in accordance with the abovementioned studies. Interestingly, we found these vancomycin susceptible *L. gasseri* strains to be resistant to clindamycin. Similar findings have been published by Danielsen and Wind (2003) and Delgado *et al*. (2005) who found clindamycin MICs equal to

or above 4µl/ml to be common for *L. gasseri*, and therefore, clindamycin resistance in *L. gasseri* could be considered as natural resistance.

It is important to confirm whether the antibiotic resistance of the probiotic strain is of intrinsic origin or is carried by highly mobile genetic elements, such as plasmids and transposons (Saarela *et al*., 2000). Plasmid-encoded erythromycin and tetracycline resistance has been reported in lactobacilli (Axelsson *et al*., 1988; Gevers *et al*., 2003). We observed no resistance to erythromycin in studied lactobacilli. These findings somewhat contradict the results of the other authors (Danielsen and Wind, 2003; Delgado *et al*., 2005) who have found some resistance to erythromycin. However, lower number of strains was used in the present study. We also observed that although most of the strains had low MICs to tetracyclines, some resistant strains appeared. These included all 4 strains of *L. plantarum* and 2 *L. oris* strains, with MICs up to 32µl/ml. According to guidelines of Scientific Committee on Animal Nutrition (SCAN) microbiological breakpoint for tetracycline is 16µl/ml for *Lactobacillus* species and strains with MICs equal to or higher than the breakpoints are considered resistant (European Commission, 2001). However, as pointed out by several authors (Zarazaga *et al*., 1999; Felten *et al*., 1999; Danielsen and Wind, 2003), there is a need for differentiating between *Lactobacillus* species when determining the breakpoints for antibiotics. Results of Danielsen and Wind (2003) showed that strains of *L. plantarum*/*pentosus* had relatively high MICs for tetracycline and they proposed 64µl/ml as a breakpoint for these species. Thus, even though the resistance of studied *L. plantarum* strains could be of intrinsic origin, the strains of both *L. plantarum* and *L. oris* should be tested further by genetic analysis and transfer experiments in order to determine whether they have transferable resistance genes. If not, the moderate natural resistance could be favorable when used in antibiotic/probiotic combination therapies.

In summary, the present study demonstrates that several human oral lactobacilli possess good antimicrobial activity as well as high tolerance of environmental stress which make them suitable for using as probiotic candidates. These properties are better expressed in facultatively heterofermentative *L. plantarum*, *L. paracasei* and *L. rhamnosus*, and homofermentative *L. salivarius*. At the same time, the strains of *L. plantarum* differ from the natural resistance pattern of lactobacilli and therefore, the absence of transferable resistance genes in these strains should be confirmed.

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Teadustöö

Peamiseks uurimisvaldkondadeks on kroonilise parodontiidi mikroobiökoloogiaga seotud probleemid ja laktobatsillide probiootilised omadused eesmärgiga otsida võimalusi infektsioonhaiguste ennetamiseks ja raviks. Avaldanud 5 teadusartiklit ja 10 konverentsiettekannet.