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Probiotic activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* in the oral cavity

An in vitro study

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ACADEMIC DISSERTATION

Abstract

The long established tradition of yogurt consumption has been related to longevity of some populations living on the Balkans. Yogurt starter *L. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus* have recently been recognized as probiotics with verified beneficial health effects. The oral cavity emerges as a relevant target for probiotic applications and probiotics have demonstrated promising results in controlling dental diseases and yeast infections. However, *L. delbrueckii* subsp. *bulgaricus* despite its broad availability in fermented dairy products has not been evaluated for possible probiotic activity in the mouth.

These series of studies were conducted to investigate *in vitro* properties of *L. delbrueckii* subsp. *bulgaricus* to outline its potential as an oral probiotic. Prerequisite probiotic properties in the oral cavity are resistance to oral defense mechanisms, adherence to saliva-coated surfaces, and inhibition of oral pathogens. *L. delbrueckii* subsp. *bulgaricus* strains showed a strain-dependent inhibition of oral streptococci and *Aggregatibacter actinomycetemcomitans*, whereas none of the dairy starter strains could affect growth of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.

Adhesion to surfaces is a factor contributing to prolonged establishment of the species at the target site. Fifteen radiolabeled dairy *L. delbrueckii* subsp. *bulgaricus* strains and *L. rhamnosus* GG were tested for their ability to adhere to saliva-coated hydroxyapatite beads and polystyrene microtiter plates. The effects of lysozyme on the adhesion of lactobacilli and of the pretreatment with lactobacilli on the adhesion of *Streptococcus sanguinis* were also assessed. The adhesion of the *L. delbrueckii* subsp. *bulgaricus* strains remained lower in comparison to *L. rhamnosus* GG. One *L. delbrueckii* subsp. *bulgaricus* strain showed binding frequency comparable to *S. sanguinis*. Lysozyme pretreatment of the samples significantly increased *Lactobacillus* adhesion to saliva-coated surfaces.

Insubstantially low gelatinolytic activity was observed in the supernatant and cell fractions of all strains supernatant specimens being slightly more proteolytic, and no conversion of proMMP-9 to its active form was induced by *L. delbrueckii* subsp. *bulgaricus*. Safety assessment ruled out deleterious effects of *L. delbrueckii* subsp. *bulgaricus* on extracellular matrix structures.

Cytokine response of oral epithelial cells after *L. delbrueckii* subsp. *bulgaricus* challenge was assessed by measuring IL-8 and TNF-α levels in cell culture supernatants. The effect of *Porphyromonas gingivalis* on cytokine secretion after lactobacilli pretreatment was also assessed. A strain- and time-dependent induction of IL-8 was observed with live bacteria inducing the highest levels of cytokine secretion. Generally, levels of TNF-α were low and only one of ten *L. delbrueckii* subsp. *bulgaricus* strains stimulated TNF-α secretion closely to that of the positive control. The addition of *P. gingivalis* produced almost an immediate reduction of cytokine levels within the first hours of incubation irrespective of lactobacilli strains co-cultured with epithelial cells.

According to this series of studies there are strains among the *L. delbrueckii* subsp. *bulgaricus* species that may have beneficial probiotic properties in the human oral cavity and their potential in prevention and management of common oral infectious diseases to be further studied.

Contents

List of original publications

This thesis is based on the following original articles referred to in the text by their Roman numerals I - IV:

I   **Stamatova I, Kari K, Meurman JH**. *In vitro* evaluation of antimicrobial activity of putative probiotic lactobacilli against oral pathogens. Int J Probiotics Prebiotics 2007;2:225-232.

II   **Stamatova I, Kari K, Vladimirov S, Meurman JH**. *In vitro* evaluation of yoghurt starter lactobacilli and *Lactobacillus rhamnosus* GG adhesion to saliva-coated surfaces. Oral Microbiol Immunol 2009;24:218-223.

III   **Stamatova I, Meurman JH, Kari K, Tervahartiala T, Sorsa T, Baltadjieva M**. Safety issues of *Lactobacillus bulgaricus* with respect to human gelatinases in vitro. FEMS Immunol Med Microbiol. 2007;51:194-200.

IV   **Stamatova I, Kari K, Meurman JH.** Probiotic challenge of oral epithelial cells *in vitro*. Int J Probiotics Prebiotics (accepted).

The publications are referred to in the text by their Roman numerals.
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Abbreviations

ATCC	American type culture collection
CFU	Ccolony forming unit
DNA	Deoxyribonucleic acid
ECM	Extra-cellular matix
EIR	Effective inhibition ratio
ELISA	Enzyme linked immunosorbent assay
GCF	Gingival crevicular fluid
GIT	Gastrointestinal tract
GRAS	Generally regarded as safe
HA	Hydroxyapatite
HDP	Host-defense peptide
IL-8	Interleukin - 8
LAB	Lactic acid bacteria
LDL	Low density lipoprotein
MRS	De Man, Rogosa and Sharpe medium
OD	Optical density
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor - α
VSC	Volatile sulphur compounds
µl	Microliter

1 Introduction

In recent decades, probiotic applications have emerged as a fascinating strategy to alleviate symptoms of various diseases, predominantly in the gastrointestinal tract. Probiotics are defined as live microorganisms conferring health benefit on the host when administered in sufficient amount (www.who.int/foodsafety/fs_management/en/probiotic _guidelines.pdf). In humans, the most frequently used probiotics are bacteria from the genera *Lactobacillus* or *Bifidobacterium*. The list of probiotic species tend to increase and new strains to be upended. The efficacy of *L. delbrueckii* subsp. *bulgaricus* as a probiotic has been questionable due to inconclusive evidence of its establishment and survival in the gastrointestinal tract. However, with the accumulation of new data and because of its ubiquitous availability in fermented dairy products, the yogurt starter *L. delbrueckii* subsp. *bulgaricus* was recognized as probiotic when a health benefit was validated in clinical trials (Guarner et al., 2005).

The oral cavity as a gateway to the underlying gastrointestinal tract is the first site of contact between probiotics and the host. Despite structural similarities with other parts of the digestive system oral cavity is unique for its highly specialized functions and characteristic site-specific pathology. The most common oral diseases with great social repercussions remain to be dental caries and periodontal disease. The infectious etiology of both these pathological conditions is well established and various strategies for control of pathogenic oral biofilms are in use.

The idea that probiotic administration may improve some disease conditions in the mouth has recently been introduced and the number of studies published gradually increase (Çaglar et al., 2005; Meurman, 2005). Among the probiotics used in the oral cavity are species such as *L. rhamnosus* GG, *L. reuteri*, *L. casei*, *B. lactis* that have shown to different extent capacity to reduce mutans streptococci counts or lessen gingival inflammation. However, the precise mechanisms explaining the observed effects yet remain unclear.

To comply with the term probiotic several basic requirements should be considered. Among these are: 1) safety of the microorganism; 2) conferring health benefits; 3) adhesion and colonization capacity; 4) inhibition of pathogens; 5) survival and resistance to human defense mechanisms. Additionally, in the scope of the oral cavity, probiotic carbohydrate and protein utilization patterns should not expose oral structures to risk of disease – such as caries.

Whenever a new probiotic candidate is evaluated a number of basic *in vitro* screening tests are used. Although *in vitro* studies have limited ability to completely reproduce authentic environmental conditions, they are essential steps in discovery of species that may further be used in clinical settings.

The millennium long tradition of yogurt consumption and the GRAS (generally regarded as safe) status of lactobacilli encouraged us to conduct this series of studies; evaluating the *L. delbrueckii* subsp. *bulgaricus* strains in its inhibitory capacity against common oral pathogens, adherence to saliva-coated surfaces, interaction with oral epithelium and above all to test its harmlessness to oral structures.

2 Review of the literature

2.1 Oral cavity in health and disease

The oral cavity as an integral part of the digestive system has various specific functions. Although it is the entry and part of the gastrointestinal tract (GIT), it possesses some distinctive features that makes it different from the rest of the digestive system. Mouth is a unique complex system of tissues and organs that together are involved in nutritional, respiratory, and communicative functions.

Oral health is important for overall health which requires harmonious functioning of several key elements in the mouth. The impact of common oral diseases extends beyond the oral cavity (Thorstensson and Johansson, 2009). Oral infection has been found to be associated with death risk in studies among middle-aged individuals (DeStefano et al., 1993; Garcia et al., 1998; Soikkonen et al., 2000; Jansson et al., 2002). The relative importance of oral health as a predictor of survival has also been analyzed and the common oral diseases have shown significant influence on survival (Österberg et al., 1990; Cabrera et al., 2005; Semba et al., 2006; Morita et al., 2006). Therefore, keeping oral health unperturbed and well balanced predisposes to long term and stable well being.

Key elements of oral homeostasis

Oral mucosa

The specific structural and functional organization of the epithelial lining of the oral cavity serves a key role in oral health maintenance. The oral epithelium provides a physical barrier to the outside world. A break in this barrier can easily lead to invasion of harmful agents into the body and particularly the exposure of the immune system to various microorganisms. Additionally, the human oral mucosa may be considered a first line of defence against invading pathogens as the oral cavity is a site where many antigens are initially encountered by the body. The role of epithelial cells has been proposed as an early warning system or sensor for infection (Eckmann et al., 2000; Aldridge et al., 2005).

The oral mucosa is anatomically divided into three tissue layers: 1) epithelium; 2) basement membrane, and 3) connective tissue. The epithelium consists of approximately 40-50 layers of stratified squamous epithelial cells. Related to its many functions, the oral cavity contains several different types of stratified squamous epithelia, including those classified as nonkeratinized, parakeratinized, and orthokeratinized (Brukhardt and Maerker, 1981). Primarily nonkeratinized epithelium provides a lining in the cheeks, lips, floor of mouth, ventral aspect of the tongue, soft palate, and upper and lower vestibular sulci. Parakeratinized and orthokeratinized epithelium lines the hard palate and the mucosa surrounding the teeth (Grafström, 2002). The major oral cell types are keratinocytes and

gingival epithelial cells (Krisanaprakornkit et al., 2000) that express specific pattern of cytokines/chemokines that distinguish them from the epithelium in the gastrointestinal tract (Formanek et al., 1999). In addition to the innate barrier function they perform, gingival epithelial cells are also capable of expressing two anti-microbial peptides of the β-defensin family, human β-defensin 1 and human β-defensin 2. The role of β-defensins has been defined in innate host defense against various oral microorganisms (Krisanaprakornkit et al., 2000, Devine, 2003, Eberhard et al., 2009; Gardner et al., 2009). Tissues of the oral cavity are constantly exposed to innate defenses derived from saliva, gingival crevicular fluid (GCF), epithelial cells, and neutrophils, and host-defense peptides (HDPs) are significant in all of these, working in synergy with other defense components.

Saliva

Saliva effectively mediates the fine coordination of various functions of the oral cavity and plays an important role in the maintenance of the overall health. Whole saliva is a complex mixture of parotid, submandibular, sublingual and minor salivary gland secretions mixed with bacteria, leukocytes, desquamated epithelial cells, and crevicular fluid (Tenovuo, 1989). Saliva is a multifunctional secretion containing components that contribute to oral buffering, lubrication, enamel mineralization, taste, digestion and aggregation (e.g. agglutinins and mucin, MUC5B and MUC7) (Nieuw Amerongen and Veerman, 2002). In addition to its flushing and clearing effect saliva with its intricate composition provides reliable defense against external irritants and contributes to the maintenance of the integrity of oral homeostasis. Constituents that are either directly antimicrobial or interfere with microbial colonization or nutrition include, for example, HDPs, secretory IgA, lactoferrin, lysozyme, sialoperoxidase, myeloperoxidase, chitinase, calprotectin, and chromagranin A (Schupbach et al., 2001; Vitorino et al., 2005; Shimada 2006).

Oral microbiota

Along with its fine structural organization the oral cavity is unique with its specific microbiota comprising an astonishing variety of species residing in oral biofilms as well as in a planktonic state in the oral fluids. The predominant genera detected in the oral cavity include *Streptococcus, Actinomyces, Veillonella, Fusobacterium, Porphyromonas, Prevotella, Treponema, Neisseria, Haemophilus, Eubacterium, Lactobacillus, Bifidobacterium, Capnocytophaga, Capnocytophaga, Peptostreptococcus, Staphilococcus*, and *Propionibacterium* (Wilson, 2005). Resident commensal populations protect tissues from colonization by exogenous pathogens, promote normal development of host cell structure and function, ensure normal development of the immune system, and coordinate immune responses (Devine and Cosseau, 2008). More than 1000 bacterial species have been identified from the human mouth (Keijser et al., 2008; Paster et al., 2006), and only 50-60% of these microorganisms can currently be cultured. A plausible explanation for

this intricacy is that some species have evolved to live within a biofilm community of interdependent species and cannot grow in monoculture (Wade, 2002; Handelsman, 2004). An investigation of the bacterial flora found in healthy volunteers showed that a given individual is colonized by 30 to 80 of the possible 1000 species at any given time (Aas et al., 2005). Within biofilms, resident bacteria gain significant advantages, that is, protection of host defenses and antimicrobial agents; expression of resistant phenotypes; and the development of food-webs and interactions such as quorum-sensing (Marsh, 2005; Roberts and Mullany, 2006; Bamford et al., 2009; Hojo et al., 2009; Keller and Costerton, 2009). The beneficial role of commensal microbiota has been evaluated in various in vitro settings indicating that some microbes can suppress epithelial cell cytokine responses (Hasegawa et al., 2007; Cosseau et al., 2008); determine normal expression of immune mediators (Dixon et al., 2004); and provide protection against colonization by exogenous microorganisms (Marsh, 2005). In general, microbial populations of the mouth are numerous, diverse and site-specific.

The oral microbiota plays critical roles in human health and is directly linked to diseases such as dental caries and periodontal diseases.

2.2 Common oral diseases

Dental caries and periodontal disease are the most common bacterial diseases of man which result from an interaction between a susceptible host, commensal microbiota and the environment. Although some specific microorganisms have been implicated in the pathogenesis of these conditions, it is now recognized that they are not classical infectious diseases but rather a complex of diseases resulting from a breakdown in the homeostasis between the human host and microbiota.

2.2.1 Dental caries

2.2.1.1 Etiology and pathogenesis

Dental caries remains one of the principal diseases in the oral cavity with a significant social impact. Caries is a result of the complex interaction between carbohydrates in food and cariogenic microorganisms in oral biofilms, influenced by the quality and quantity of saliva, and clinically manifested by demineralization and destruction of dental hard tissues. Recent development in molecular analyses have shown that all the bacteria that have been associated with caries belong to the normal microbiota of the oral cavity and dental caries is regarded as an endogenous infection (Fejerskov and Nyvad, 2003; Takahashi and Nyvad, 2008). Three major hypotheses for the etiology of caries have been supported: the specific plaque hypothesis, the non-specific plaque hypothesis, and the ecological plaque hypothesis (Loesche 1992; Marsh 1994; Martin et al., 2002). In light of

the ecological plaque hypothesis caries is a result of a shift in the balance of resident microbiota driven by changes in local environmental conditions (Aas et al., 2008). It is generally believed that all three parameters (microorganisms, the host, and environment) must "act" simultaneously for carious lesions to develop and progress and to become visually detectable (Shaw et al., 2008).

A wide group of microorganisms are identified from carious lesions of which *Streptococcus mutans*, *Lactobacillus acidophilus*, and *Actinomyces viscosus* may be considered the main pathogenic species involved in the initiation and development of dental caries (Shivakumar et al., 2009). *Streptococcus mutans*, initially isolated in 1924, has been primarily implicated in this disease (Hamada and Slade, 1980; Loesche, 1986) and extensively studied throughout several decades. Some significant virulent traits of *S. mutans* that contribute to caries initiation and progression are: (i) initiation of biofilm formation by adherence and accumulation on the tooth surface that is promoted by its synthesis of insoluble, extracellular polysaccharides; (ii) production of numerous bacteriocins that kill other species, favouring its competition in dental biofilms; (iii) high efficiency in catabolizing carbohydrates and producing acids; and (iv) the ability to tolerate low pH (Belli and Marquis, 1991; Li and Burne, 2001; Kuramitsu, 2003; Scheie and Petersen, 2004). Various studies have shown that the expression of virulence traits by *S. mutans* requires multiple signal transduction pathways and complex regulatory networks. A signal peptide-mediated quorum-sensing system encoded by *com*CDE genes has been found to play a central role in regulation of genetic competence, bacteriocin production, biofilm formation and stress response (Li et al., 2001a, b, 2002a; van der Ploeg, 2005). Additionally, the genes that appear to be important for the cariogenicity of *S. mutans*, are regulated at transcription level (Jayaraman et al., 1997; Hiratsuka et al., 1998). Although numerous *in vitro* studies provide evidence of molecular mechanisms of *S. mutans* cariogenicity and this species appears the most extensively studied, *in vivo* test models do not generally validate basic laboratory findings. For example, Aas et al., (2008) have demonstrated that 10% of subjects with rampant caries do not have measurable levels of *S. mutans*. No detectable levels of *S. mutans* were also reported in 10 to 15 % of caries-active subjects, thus indicating that the presence of *S. mutans* does not necessarily reflect caries activity (Beighton 2005). Furthermore, phenotype of a bacterium expressed in laboratory culture may not represent the properties expressed by the same organism *in vivo*.

Key findings in the diversity of oral microbial species during past 10 years have changed the view of the etiology of caries. Molecular biology techniques have shown that more than 50% of the oral species are uncultivable by conventional methods (ten Cate 2009). It is now recognized that caries results not solely because of the presence of *S. mutans* or any single organism in dental plaque, but it is rather the interaction of multiple acid-producing organisms such as low-pH non-mutans streptococci, *Veilonella*, *Lactobacillus, Propionibacterium, Bifidobacterium* that may be involved in the initiation of the disease (Aas et al., 2008; He et al., 2009; Matzourani et al., 2009). The ecological plaque hypothesis suggests that the cariogenic oral environment will select for increased proportions and numbers of acidogenic and aciduric microbiota with certain taxa exhibiting a reduced presence under these conditions (Matzourani et al., 2009).

2.2.1.2 Treatment and prevention

The classical treatment plan for caries yet remains to be the operative approach of complete caries removal. A series of novel methods of caries removal have been described; including chemomechanical caries preparation, air abrasion, sono-abrasion, polymer rotary burs and lasers (Ricketts and Pitts, 2009). However, more scientific efforts are directed towards discovering effective methods for caries prophylaxis based on inhibiting the known mechanisms of caries development. The elimination of cariogenic bacteria from the oral cavity using antibacterial agents is one of the primary strategies for the prevention (Wicht et al., 2003; Caufiled, 2005; Altman et al., 2006; Modesto and Drake, 2006; Johansson et al., 2008). Fluoride treatment used worldwide has successfully limited caries progression, but was not sufficient to control this infectious disease even when used together with professional tooth cleaning and dietary counselling in populations exposed to cariogenic microbiota (Haugejorden and Birkeland, 2005; Yee et al., 2006; Akers, 2008; Carvalho et al., 2009). Polyphenols from plant stimulant beverages like cocoa, coffee, and tea have shown pronounced antimicrobial effect against *S. mutans*, and can additionally be implemented in the prevention of pathogenesis of dental caries (Ferrazzano et al., 2009). Polyphenols in stimulant beverages significantly reduce biofilm formation and acid production by *S. mutans* and *S. sanguinis*. Further, as an example, *in vitro* studies have shown that *S. mutans* is susceptible to methanol and aqueous extracts of *Garcinia kola, Hibiscus sabdariffa* (Afolabi et al., 2008).

Sugar substitutes have a long history of being effective in caries reduction. The main sugar substitutes used are sorbitol and xylitol. Xylitol is not fermented by oral bacteria and is considered to be non-cariogenic while sorbitol in solution can be fermented slowly by mutant streptococci. Chewing sorbitol-sweetened gum does not cause a fall in plaque pH, however (Edgar, 1998). A regular consumption of xylitol lozenges can modify dental plaque resulting in marked reduction in the plaque acidogenicity (Splieth et al., 2009).

Active and passive immunization strategies which target key elements in the molecular pathogenesis of mutans streptococci hold promise. Considerable caries reduction could be attained if colonization of *S. mutans* could be prevented or reduced at the time of eruption of both deciduous and permanent teeth. Thus, a successful vaccination directed against *S. mutans* could be a valuable adjunct to other caries-preventive measures. However, *S. mutans* being the sole target species in caries prophylaxis does not comply with the key principles of ecological plaque hypothesis.

Bacteriotherapy further emerges as a fascinating approach in oral infectious disease management. A daily application of JH145, a naturally occurring LDH-deficient variant of *S. rattus*, could compete with *S. mutans* for its habitat on the tooth surface and thus contribute to caries prevention (Hillman et al., 2009).

2.2.2 Periodontal disease

2.2.2.1 Etiology and pathogenesis

Periodontal disease has been described as "a heterogeneous group of pathoses characterized by a predominance of specific infectious agents in the face of inadequate local host defenses" (Slots, 2005). The definition reflects the complexity of periodontal disease.

A primary risk factor considered in the etiology and progression of periodontal disease is the infection by specific bacterial pathogens. The actions of bacterial virulence factors, directly or indirectly through the activation of the immune system, cause swelling, inflammation, and gingival pocket formation. The balance between protective and destructive immune responses is a key determinant of disease progression. This balance is strongly influenced by the host response to the challenge caused by subgingival bacteria (Sakamoto et al., 2005; Teng, 2006a, Teng, 2006b). Socransky et al., (1998) have formulated a color coded complex for periodontal pathogens with respect to their destructive potential. The "Red complex", which includes *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, strongly correlated to chronic periodontal disease, and the first two species together with *Aggregatibacter actinomycetemcomitans* are currently recognized as the main causative species of periodontal disease (Borrell and Papapanou, 2005; Nørskov- Lauritsen and Kilian, 2006).

Destruction of the periodontal ligament and resorption of the alveolar bone leading to tooth loss is the hallmark of periodontal disease. Since host and microbiota interactions are dynamic, disease may arise at the mucosal surface of a susceptible host when a perturbation occurs in the epithelial environment, for example, when the host becomes immunocompromised, or as a result of the unintended (in an evolutionary sense) consequences of bacterial activity (Galan and Zhou, 2000). The initial interface between the host and the potentially periodontopathic organisms, such as *P. gingivalis* and *A. actinomycetemcomitans*, is the epithelial layer that lines the subgingival crevice. Epithelial cells are both a physical barrier to infection and a component of a network that efficiently signals microbial intrusion to the immune cells to insure effective mobilization of the innate and specific defense mechanisms (Kagnoff and Eckmann, 1997). Studies have shown that several periodontal pathogens, *A. actinomycetemcomitans*, *P. gingivalis, F. nucleatum,* and *T. denticola*, can effectively invade and exist in oral epithelial cells (Sreenivasan et al., 1993; Rudney et al., 2005; Vitkov et al., 2005; Sakakibara et al., 2007). Furthermore, intracellular *P. gingivalis* is able to inhibit apoptosis, a feature that may contribute to bacterial persistence and chronic, slowly progressing tissue destruction (Nakhjiri et al., 2001). Localization of bacteria in host tissues provides an ideal position from which the microorganism can effectively deliver toxic molecules and enzymes and at the same time can avoid host defense mechanisms. For example, *P. gingivalis* is able to inhibit production of IL-8 by epithelial cells, which may provide the microorganism with an advantage in evading polymorphonuclear(PMN)-mediated killing (Dareveau et al., 1998).

17

Host tissue damage can be due to bacterial properties resulting directly in degradation of host tissues and those causing release of biologic mediators from host tissue cells that lead to tissue destruction. A large group of enzymes produced by periodontal microorganisms appear capable of degrading host tissues and intercellular matrix molecules. Bacterial products may perturb the immune system resulting in tissue destruction. The proportion of *P. gingivalis, A. actinomycetemcomitans, T. forsythia* and the number of CD4(+) T are higher in active than in inactive sites (Silva et al., 2008).

Pathologically increased activity of matrix metalloproteinases (MMP-2; MMP-8; MMP-9; MMP-13) in inflamed periodontal structures leads to periodontal destruction due to collagen degradation (Biyikoğlu et al., 2009; Gu et al., 2009; Marcaccini et al., 2009; Yamazaki-Kubota et al., 2009). Furthermore, some periodontal pathogens may indirectly contribute to tissue damage by induction of host tissue proteinases such as elastase and MMPs (Pattamapun et al., 2003; Tiranathanagul et al., 2004; Bodet et al., 2007; Guam et al., 2008). *A. actinomycetemcomitans* and *P.gingivalis* can elevate MMP-2 secretion in human periodontal ligament fibroblasts (PDLFs), indicating that periodontal pathogens play an important role in tissue destruction and disintegration of extracellular matrix in periodontal diseases (Chang et al., 2002).

2.2.2.2 Treatment and prevention

Traditionally periodontal disease treatment is a four-phase approach including non-surgical periodontal therapy, surgical procedures, restorative treatment, and supportive care or maintenance. The foremost goal in periodontal therapy is the elimination or reduction of the pathogenic potential of dental plaque. However, even with appropriate treatment and improved oral hygiene many patients fail to respond to therapy unless certain factors (e.g. smoking, uncontrolled diabetes) are also eliminated.

One strategy to prevent periodontal disease may be the controlling of factors that disrupt the microbial ecological balance from a symbiotic and healthy to a host-pathogen relationship which then leads to disease (Kinane et al., 1999). Systemic and local antibiotic applications have been used as adjunct to conventional periodontal therapy. However, because of the chronic nature of periodontitis antibiotic medications are not generally used except in patients who do not respond to conventional therapy. Inappropriate use of antibiotic agents can lead to overgrowth of potentially pathogenic organisms and development of bacterial drug resistance.

A novel prophylactic strategy in periodontal disease management that merits further investigations is the replacement of common periodontal pathogens by commensal oral microbes. Teughels and coworkers have tested the hypothesis that the subgingival application of *S. sanguinis* KTH-4, *S. salivarius* TOVE and *S. mitis* BMS after mechanical debridement would enhance the microbial shift away from periodontopathogens (Teughels et al., 2007). A significant delay in recolonization of periodontal pockets by *A. actinomycetemcomitans, P. gingivalis, P. intermedia*, and *Tannerella forsythia* after root planing was observed when the above species were locally applied.

Scientific understanding of molecular mechanisms in the development and progression of common oral diseases can foster the implementation of natural host defense mechanisms to combat oral infections. The application of "health-promoting" bacteria for therapeutic purposes is one interesting field in this regard.

2.3 Probiotics from the oral health perspective

2.3.1 Probiotics

2.3.1.1 Emergence and definition of the term

The word probiotic is derived from the Greek "probiosis" meaning "for life" and generally applies to bacteria causing no harm to the host. The probiotic concept dates back over 100 years, and associates with the name of the Ukranian bacteriologist and Nobel Laureate Ilie Metchnikoff, who proposed the scientific rationale for the beneficial effects of lactic acid bacteria. In 1888 while working in the Pasteur Institute in Paris, Ilie Metchnikoff emphasized a theory that putrefactive-type fermentation products by some gut pathogens may be the cause for autointoxication of the macroorganism. Furthermore, the intake of bacteria involved in yogurt fermentation, *L. bulgaricus* and *Streptococcus thermophilus*, can effectively suppress metabolic activity of pathogenic intestinal species thus maintaining health. He claimed that the longevity of some populations in Bulgaria, Turkey and Armenia was due to regular consumption of fermented milk products rich in live lactic acid bacteria (LAB). The scientist has promoted the idea that LAB in yogurt may neutralize deleterious effects of gut pathogens, thus extending life span. He further contributed to the adoption of the name of the species, *L. bulgaricus*, one of the essential yogurt starter microorganisms. This also meant the birth of modern dairy industry (Meurman, 2005).

Despite inconclusive evidence of health effect of yogurt bacteria research interest intensified in the later years. Ferdinand Vergin was the first (1954) to introduce the term "probiotic" mainly opposing it to antibiotics. Kollath (1954) used the term to designate "active substances that are essential for healthy development of life". Lilly and Stillwell (1965) contributed to the adoption of probiotics as scientific term providing evidence that bacteria secrete substances that stimulate the growth of another. The definition underwent further modifications broadening its meaning. Parker (1974) defined them as: "organisms and substances which contribute to intestinal balance". The closest to contemporary meaning of probiotics has been given by Roy Fuller (1989): "a live microbial feed supplement which beneficially affects the host animal by improving the intestinal microbial balance". Yet probiotic activity was limited to live bacterial species. However, accumulation of new research data contributed to the understanding of probiotic activities beyond the scope of gastrointestinal tract and bacterial cell fractions were also claimed

effective. Although the concept of probiotics remains open to further modifications, in 2002 the Joint Food and Agriculture Organization/World Health Organization Working Group (FAO/WHO) officially formulated the term "probiotics": "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". This definition was adopted by the International Scientific Association for Probiotics and Prebiotics (Reid et al., 2003). The definition retains the historical elements of the use of living organisms for health purposes but does not restrict the application of the term only to oral probiotics with intestinal outcomes.

Probiotics can also target the oral cavity, nasopharynx, stomach, vagina, bladder and skin. Another implication of the FAO/WHO definition is that unless strains are shown to confer clinically established physiological benefits, they should not be referred to as probiotics (Reid et al., 2003). Under the formulation of the latter definition probiotics are linked to food and to food only, thus excluding any reference to the term "biotherapeutic agents". However, emerging data suggest that while viable organisms may be most effective for specific effects, non-viable probiotic organisms (abiotics) may be efficacious in specific situations (Salminen et al., 1999; Shortt, 1999). It is likely that the abiotic idea, if accepted, will further broaden the health potential of the probiotic concept in the future (Shortt, 1999).

The principal microorganisms in use as probiotics belong to the genera *Lactobacillus* and *Bifidobacterium.* However, other genera including *Escherichia, Enterococcus*, and *Saccharomyces* are also used. Lactobacilli and bifidobacteria constitute the two most important probiotic groups under consideration owing to their recognition as members of the indigenous microbiota of humans, their history of safety and the general body of evidence that supports their positive roles. At this stage, phylogenetics has recognized 97 species of lactobacilli (Dellaglio and Felis, 2005), 18 of which are considered to be of some interest in probiotics; and 31 species of *Bifidobacterium*, 11 of which have been detected in human feces (Sanders, 1999). Lactic acid bacteria are associated with habitats that are rich in nutrients, such as various food products. They can be found in soil, water, sewage, and they can ferment or spoil food. Certain LAB species are inhabitants of the human oral cavity, the intestinal tract, and the vagina, and may have a beneficial influence on these human ecosystems (Holzapfel et al., 2001).

2.3.1.2 Beneficial effects of probiotics

Numerous health benefits have been proposed to result from consumption of probiotic bacteria. Although the specific mechanisms involved in the many suggested benefits have not been completely established, evidence suggests that probiotics can influence various disease conditions in a positive manner. *Table 1* outlines the most common clinical conditions with a positive outcome after probiotic administration.

Table 1. Clinical conditions improved by probiotic intake

Disorder	Probiotic	Patient group	Duration	Clinical effect	Reference
GI disorder					
Ulcerative colitis	*E. coli* Nissle 1917	116	12 months	Induction of remission; prevention of relapses	Rembacken et al., 1997
	E. coli Nissle 1917	120	12 weeks	Maintaining the remission	Kruis et al., 2004
	B. longum	120	4 weeks	Improved systemic function	Fujimori et al., 2009
	VSL#3	29	12 months	Remission maintenance	Miele et al., 2009
	L. rhamnosus GG	187	12 months	Prolongation of relapse-free time	Zocco et al., 2006
	E. coli Nissle 1917	327	12 months	Induction of remission	Kruis et al., 2001
	Saccharomyces boulardii	25	4 weeks	Induction of remission	Guslandi et al., 2003
Crohn`s disease	*Saccharomyces boulardii*	34	3 months	Improved intestinal permeability	Garcia Vilela et al., 2008
	L. johnsonii	98	6 months	Postsurgical Crohn`s disease recurrence	Marteau et al., 2006
	E. coli Nissle 1917	24	3 months	Relapse rate decreased	Guslandi et al., 2000
	Genetically	10	7 days	Decrease in	Braat et al.,

	modified *L. lactis* (LL Thy12) delivering IL-10			disease activity	2006
Pouchitis	VSL#3	36	12 months	Maintaining the remission	Mimura et al., 2004
	VSL#3	23	4 weeks	Prolongation of remission	Gionchetti et al., 2007
Lactose maldigestion	*L. acidophilus*	20	On intake	Decreased symptoms of lactose-maldigestion	Montes et al., 1995
Diarrhea episodes	*L. rhamnsosus GG*	204	15 months	Reduction of diarrhea episodes in children	Oberhelman et al., 1999
	L. rhamnosus 19070-2; L. reuteri DSM 12246	69	5 days	Reduction of diarrhea phase	Rosenfeldt et al., 2002
	L. paracasei ST 11	230	5 days	Improved management of non-rotavirus diarrhea	Sarker et al., 2005
	L.rhamnosus GG	140	5 days	Shorten diarrhea duration	Guandalini et al., 2000
	Probiotic combination	75	5 days	Shorten diarrhea periods	Teran et al., 2009
Allergy states	*L. acidophilus* NCFM*; B. lactis*	47	4 months	Prevention of pollen-induced infiltration of eosinophils	Ouwehand et al., 2009

Lactobacillus F19	89	7 months	Prevents early manifestation of allergy	West et al., 2009
L.GG; L.gasseri TMC*0365*	40	10 weeks	Decreased allergic rhinitis symptoms	Kawase et al., 2009

Mechanisms of action explaining the beneficial probiotic effects, though still unclear, may include the modulation of host immune response leading to strengthening of the resistance to pathogenic challenge; alteration of the composition and metabolic activity of host microbiota at the specific location; interference with pathogen adhesion and growth inhibition (Hatakka and Saxelin, 2008).

2.3.1.3 Selection criteria for probiotic candidates

A wide range of requirements have been discussed as related to various applications of probiotics (Lee, 2009), but among key selection criteria with emphasis on human health are:

- Adhesion and colonization (at least transitory) of human body. Adhesion may increase the retention time of a probiotic and place bacteria and host surfaces (body fluids and epithelial cells) in close contact thus facilitating further probiotic activity;
- Enhancement of the non-specific and specific immune response of the host;
- Production of antimicrobial substances and competition with pathogens for binding sites;
- Survival and resistance to human defense mechanisms during the oro-gastro-intestinal transit;
- Human safety.

Additionally, the probiotic candidate should (1) be of human origin; (2) be non pathogenic; (3) confer clinically established physiological benefits; and (4) maintain viability and activity throughout product manufacture and processing (Dunne et al., 1999; Reid et al., 2003). Generally the list of criteria for probiotic selection is application-based and depends on the specific probiotic effects desired and the target site of action.

Oral cavity with its complex anatomy with both soft and hard tissues and functional integrity is a new area for studies of probiotic therapy in the treatment and prevention of most common disorders in the mouth. Species investigated from an oral health perspective are given in *Table 2*.

Table 2*. Probiotic candidates for the oral cavity*

Lactobacillus	*Bifidobacterium*	*Streptococcus*	*Propionibacterium*	*Weissella*
L. reuteri	*B. lactis*	*S. salivarius K12*	*P. freudenreichii*	*W. cibaria*
L. plantarum	*B. longum*	*S. thermophilus*		
L. rhamnosus	*B. infantis*			
L. salivarius				
L. acidophilus				
L. casei				
L. johnsonii				

Among the selection criteria with relevance of probiotics to the mouth are:
- Attachment, adhesion, and oral colonization;
- Resistance to oral defence mechanisms;
- Production of antimicrobial substances and competition with pathogens for binding sites;
- Carbohydrate and protein utilization patterns;
- Enhancement of the non-specific and specific immune response of the host;
- Safety to oral ecology and oral structures.

2.3.1.3.1 Attachment, adhesion, and oral colonization

The evidence is scarce regarding the question whether probiotics permanently reside in the human body and in the mouth, in particular (Petti et al., 2001; Yli-Knuuttila et al., 2006). However, it can be anticipated that among the $10^3 - 10^4$ CFU/g lactobacilli found in the oral cavity (Bernardeau et al., 2008) there are species/strains capable of exerting probiotic properties. Bacteria reside in the mouth either in planktonic state or are finely integrated in biofilm on various oral surfaces. Oral biofilms are dynamically changing and develop increasingly complex structures as they mature. Interaction between species is characteristic in biofilms. Some species may depend on others to provide favorite environment for colonization. Furthermore, bacteria in biofilms differ physiologically from their planktonic counterparts and tend to be much more resistant to environmental factors and antimicrobial agents. It has been established that distinct genes become active when planktonic bacteria bind to surfaces and grow in biofilms (Burne et al., 1999; Rudney, 2000). On the other hand, saliva is the essential medium in the mouth contributing to the microbial diversity. It plays an integral role in propagating oral biofilms. Salivary flow can easily lead to detachment of some microbes from biofilm

surfaces and thus modulate microbial colonization. Furthermore, as complex medium saliva contains different proteins with bactericidal, bacteriostatic, or inhibitory activity that collectively may affect a variety of species in planktonic state (Germaine and Tellefson, 1986; Rudney et al., 1991; Hahnel et al., 2008; Grölsch et al., 2009). Biofilm species composition can also depend on phenomena like auto- or co-aggregation that may prevent microorganisms from establishing themselves in the biofilms. Hence by taking into consideration the multifaceted nature of biofilm development and multivariate species interactions we can acquire better understanding and interpretation of studies with probiotics in the oral cavity.

There are very few studies of the colonization of probiotic bacteria in the oral cavity, and the results are contradictory. The pattern of oral colonization by probiotic species has been found to be transient and gradually diminished soon after probiotic administration period ended (Bussscher et al., 1999; Petti et al., 2001; Yli-Knuuttila et al., 2006). Therefore, it is necessary to consider that the associations and mechanisms less intricate and more transient than those of native microflora may mediate probiotic effects. On the other hand probiotic administration early in life may provide those species the opportunity to interact with host receptors early and subsequently integrate in microbial communities resulting in permanent condition. In a study comparing species variability in the mouth and feces Ahrne et al. (1998) have discovered that species most frequently recovered from the rectal as well as from the oral mucosa were *L. plantarum* and *L. rhamnosus*, which were present in 52% and 26% of the individuals, respectively. However, this study did not define those strains as permanent colonizers of the two sites tested or whether the mouth is their natural habitat. The most common species of lactobacilli recovered from saliva of a Thai population were *L. fermentum* and *L. rhamnsous* (Teanpaisan et al., 2006). A promising finding was that lactobacilli population differed between healthy individuals and those with periodontal disease. Koll-Klais et al. (2005) have observed that healthy persons are populated by *L. gasseri* and *L. fermentum*, whereas the predominant species in periodontitis patients was *L. plantarum* while the first two were undetectable. Observations by this study group showed that microorganisms with probiotic properties may indeed exist and reside in the oral cavity. However, the complexity of biofilm development and interspecies interactions require more thorough investigations in order to assert true probiotic candidates with activity in the oral cavity.

The mechanism of adhesion to oral surfaces is an issue of importance for the long term probiotic effect. The capacity of probiotics to adhere to surfaces of the oral cavity can avoid or at least reduce rapid clearance from the environment. Among the different assays available to study the adhesion phenomenon, two model systems predominate: systems using saliva-coated hydroxylapatite (HA) and hydroxylapatite coated with buffers, proteins, and other substances (Ostengo and Nader-Macias, 2004). Probiotics and putative probiotic strains have been shown to vary extensively in their adhesiveness to saliva-coated surfaces. Lactobacilli have shown better adherence than bifidobacteria to saliva-coated hydroxyapatite beads and polystyrene plates (Haukioja et al., 2006). Furthermore, interplay between saliva and probiotics may additionally modify composition of salivary pellicle thus altering the attachment pattern. *In vitro* removal of a heavy molecular weight protein band that contained salivary agglutinin gp340 has been observed after incubation

of saliva with four commercially available probiotic strains (Haukioja et al., 2008). The mechanisms of adhesion of lactobacilli involve hydrophobicity and surface charge, as well as specific carbohydrate and/or proteinaceous components (Lorca et al., 2002). The interaction between bacterial and HA surfaces have been shown to depend not only on the nature and number of available anchoring groups, but also on the calcium ions in the medium that bind the functional groups of the bacteria to the biomaterial (Venegas et al., 2006).

The adhesion of probiotic bacteria to oral soft tissues is another aspect that promotes their health effect to the host. Cell adhesion is a complex process involving contact between the bacterial cell and interacting surfaces. Secretome studies can provide valuable information about bacterial structures responsible for binding to host surfaces. The domain composition of the *L. plantarum* proteins predicted appeared to be involved in the adherence to extracellular macromolecules (Boekhorst et al., 2006).

2.3.1.3.2 Resistance to oral defence mechanisms

It is generally considered that to be able to exert its beneficial effect the probiotic candidate should survive the oro-gastrointestinal passage. Ingested probiotics are exposed first to saliva which mediates the contact with hard and soft oral tissues. During this first step of contact with tissues resistance to environmental factors in the mouth are of paramount importance for bacteria to survive. Salivary proteins such as lysozyme, lactoferrin, histatin, salivary peroxidase, cystatins, and secretory IgA can collectively affect viability or cell surface morphology of probiotic species and further affect their adhesion and metabolic activity. Saliva can kill or damage species in planktonic state as well as mediate intra- and interspecies aggregation, thus additionally affecting adhesion. The role of saliva on microbial establishment can be contradictory, however, inhibiting colonization on one hand (by growth inhibition, killing, or prevention of adherence to host tissues), and promoting microbial colonization, on the other hand (Bosch et al., 2003). *In vitro* studies testing probiotic survival in saliva have shown that *Lactobacillus* and *Bifidobacterium* strains cannot grow in saliva but remain viable after 24 hours of incubation (Haukioja et al., 2006). Lysozyme pretreatment has been observed to significantly reduce the adhesion of *L. rhamnosus* GG, *L. rhamnosus* Lc705 and *L. casei* Shirota. However, the adhesive properties of *L. johnsonii* La1 and *B. lactis* Bb12 remained unaffected. These results emphasize the strain-specific response to proteolytic enzymes and this feature needs to be considered when selecting probiotics for the oral cavity.

2.3.2 Clinical relevance of probiotics in the oral cavity

Probiotic relevance in the management of common oral diseases has been advocated in a number of clinical studies. ***Table 3*** lists the species/strains that have been observed to positively affect infectious oral diseases.

Table 3. Clincal trials with positive effect after probiotic administration

Oral disease	Probiotic	Vehicle of administration	Duration of the study	Result	Reference
Dental caries	*L. rhamnosus* GG	Cheese	3 weeks	Reduction of *S. mutans*	Ahola et al., 2002
	L. rhamnosus GG	Milk	7 months	Lower *S. mutans* counts	Näse et al., 2001
	Bifidobacterium DN-173010	Yogurt	4 weeks	Reduction of *S. mutans*	Çaglar et al., 2005
	B. animalis subsp. *lactis* DN-173010	Fruit yogurt	4 weeks	Reduction of *S. mutans*	Cildir et al., 2009
Gingivitis and periodontitis	*L. reuteri*	Chewing tablet	2 weeks	Improved gingival health and reduced plaque accumulation	Krasse et al., 2006
	L. reuteri	Chewing gum	2 weeks	Improved bleeding on probing, and decrease of GCF volume	Twetman et al., 2009
	L. casei 37	Periodontal dressing	Several days in periodontal dressing	Reduction of periodontal pathogens	Volozhin et al., 2004
C. albicans infections	*L. rhamnosus* GG; *P.*	Cheese	16 weeks	Decreased prevalence	Hatakka et al.,

27

freudenreichii ssp.*shermanii* JS	of *C.albicans*	2006

2.3.2.1 Probiotics and dental caries

The first randomized, double-blind, placebo-controlled intervention study of *L. rhamnosus* GG effect on dental caries was completed in 2001; the study included 594 children, 1-6 years old, who consumed milk for 7 months (Näse et al., 2001). Probiotic milk was able to reduce *S. mutans* counts at the end of the trial and a significant reduction of caries risk was also observed. *S. mutans* reduction was also achieved after consumption of *L. rhamnosus* LC 705, *Bifidobacterium* DN-173 010, and *B. lactis* Bb-12 in cheese, yogurt, or ice-cream as vehicles (Ahola et al., 2002; Çaglar et al., 2005; Çaglar et al., 2008). Fermented dairy products being a favorable habitat for lactobacilli are generally used as vehicles for probiotic administration. However, for the scope of the oral cavity several other means of administration have been assessed. A tablet, a telescopic straw, a lozenge, and a chewing gum containing probiotics have shown reduction of common caries pathogen after 3-week regular intake (Çaglar et al., 2006; Çaglar et al., 2007; Çaglar et al., 2008). The observed positive correlation between probiotic intake and reduction in caries pathogen counts might be a useful strategy in caries prophylaxis for some special risk groups. For example, orthodontic patients wearing fixed appliances can experience higher caries risk during treatment. Cildir et al. (2009) have shown that a probiotic intake of *B. animalis* subsp. *lactis* DN-173010 can positively reduce salivary mutans streptococci in orthodontic patients.

Possible explanation for the clinical results of probiotic intake may be the competition for binding sites in oral biofilms as shown in some *in vitro* studies (Haukioja et al., 2008). However, this area also calls for more in-depth studies.

2.3.2.2 Probiotics and periodontal disease

Only few clinical studies outlining probiotic effectiveness in periodontal disease have been published to date. From the periodontal health perspective it should be noted that the composition of lactobacilli species differs in healthy and periodontitis patients and that obligately homofermentatives are less prevalent in chronic periodontitis (Koll-Klais et al., 2005). A fourteen-day intake of *L. reuteri* led to the establishment of the strain in the oral cavity and significant reduction of gingivitis and plaque in patients with moderate to severe gingivitis (Krasse et al., 2006). Salivary inflammatory markers of periodontal disease can be positively affected in smokers after *L. salivarius* WB21 tablet form administration for eight weeks (Shimauchi et al., 2008). Periodontal inflammation has been reduced after the intake of probiotic tablets (Bifidumbacterin and Acilact) available

on the Russian market (Grudianov et al., 2002). Studies from Russia have also shown that a periodontal dressing containing *L. casei* 37 can reduce the number of most common periodontal pathogens and extend remission up to 10 – 12 months (Volozhin et al., 2004). Possible explanation to the results might be the inhibitory effect of probiotics on pathogen growth thus altering the composition of oral biofilm. Due to its ability to inhibit *P. gingivalis, L. salivarius* TI 2711 was given for 4 or 8 weeks in a tablet to healthy volunteers at a concentration of $2x10^7$ CFU/ml. A significant reduction of blackpigmented rods in saliva was observed (Ishikawa et al., 2003). Additional finding in this study was the increase of pH to neutral after treatment, thus highlighting both caries and periodonto-prophylactic properties. The effectiveness of the latter *Lactobacillus* strain has been confirmed by Matsuoka et al., (2006).

A proposed mechanism of action of probiotics is strengthening the mucosal barrier via tropic effects on the epithelium and stimulating both the innate and adaptive immune response. A double-blind, placebo-controlled clinical trial with *L. reuteri* ATCC 55730 and ATCC 5289 taken in a chewing gum for 10 min twice daily has shown reduction of pro-inflammatory cytokines TNF-α and IL-8 in gingival crevicular fluid (Twetman et al., 2009).

Because of the broad diversity of species residing in the mouth new probiotic candidates may be anticipated to emerge adding to the array of already known strains. A novel concept favoring periodontal health has been introduced by Teughels and co-workers (Teughels et al., 2007; Nackaerts et al., 2008) suggesting re-colonization of the gingival pocket after scaling and root planning by species like *S. sanguinis* KTH-4, *S. salivarius* TOVE and *S. mitis* BMS these strains then thought to be able to inhibit adhesion of common periodontal pathogens. The foundation of the re-colonization concept stands on the principle that subgingival application of oral streptococci would enhance the microbial shift away from periodontopathogens.

2.3.2.3 Probiotics and other oral disorders

Among other oral conditions that may be favorably affected by probiotic administration are *Candida* infections and halitosis.

Halitosis, foetor ex ore, has mainly been ascribed to the production of volatile sulfur compounds (VSC) by Gram negative anaerobes residing in periodontal pockets and on the tongue dorsum. Halitosis has been significantly improved in subjects after probiotic intake. *S. salivarius* K12 taken in a lozenge after a mouth wash led to reduction of VSC levels in 85% of the subjects (Burton et al., 2006). Furthermore, *L. salivarius* has been the most prominent species detected in healthy subjects, whereas in individuals with halitosis it was almost undetectable or only at very low levels (Kazor et al., 2003). *W. cibaria* is another species with probiotic properties which has been shown to reduce VSC production both *in vitro* and *in vivo* (Kang et al., 2006). A contributing factor to the malodor reduction can be the ability of *W. cibaria* to co-aggregate with species known for their VSC production (*F. nucleatum*, for example). The aggregation thus affects the source for malodorous compounds in the oral cavity (Kang et al., 2005).

Candida albicans is the commonest pathogen of oral fungal infections. Probiotic applications may alleviate symptoms and reduce pathogenic potential of *Candida* species. A 16-week probiotic intervention study demonstrated a significant reduction by 75% of high yeast counts in the elderly (Hatakka et al., 2007). The intake of *L. rhamnosus* GG containing cheese associated with control of oral *Candida* also led to reduction of the risk of hyposalivation as reported by the same authors. Although this is the only study published on the role of probiotics on yeast infection in humans two other *in vivo* studies on mice have shown that lactobacilli might indeed be effective in controlling oral candidiasis. Elahi et al., (2005) have demonstrated a higher clearance of *C. albicans* in mice fed with *L. acidophilus* compared to the control group. However, in another study no noticeable delay in colonization of the oral cavity by *C. albicans* of immunocompromized mice was achieved when heat killed *L. casei* and *L. acidophilus* cells were given (Wagner et al., 2000).

2.4 *L. delbrueckii* subsp. *bulgaricus* as a probiotic

The discovery of *Lactobacillus bulgaricus* relates to Stamen Grigorov, a Bulgarian microbiologist who in 1905, in the laboratory of Professor Masole in Geneva, isolated the species from yogurt and thereafter the microorganism was named after the country. "*Lactobacillus bulgaricus*" was formally described by Orla-Jensen in 1919 and validated in 1971 with the study of Rogosa and Hansen (1971). After a number of different studies, Weiss et al. (1984) proposed the union of *Lactobacillus delbrueckii, Lactobacillus leichmannii, Lactobacillus lactis* and *Lactobacillus bulgaricus* under the name of *L. delbrueckii*, and thereafter the name of the former "*Lactobacillus bulgaricus*" was changed in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Within the species, three subspecies were recognized to exhibit DNA-DNA homologies of 90-100% among each other (Howey et al., 1990; Torriani et al., 1997; Germond et al., 2003). Consequently, they cannot be easily identified, not even by molecular methods, and can be mistakenly confused if only the phenotypic characteristics are known (Milliere et al., 1996; Vandamme et al., 1996; Giraffa et al., 2003).

L. delbrueckii subsp. *bulgaricus* is a Gram-positive, non-motile, obligatory homofermentative, catalase-negative rod (*Figure 1*). Its DNA has 49-51 % GC ratio (Hammes and Vogel, 1995), which is significantly higher compared to the GC content of other lactobacilli in the genus (Nicolas et al., 2007). Carbohydrate fermentation results in 99.5 % D- and 0.5 % L-lactic acid. *L. bulgaricus* encodes many partial carbohydrate metabolic pathways and shows a distinct preference for growth in lactose rich media. It maintains extensive proteolytic and amino acid transport systems which are useful in the protein-rich milk environment (Klaenhammer et al., 2008). *L. delbrueckii* subsp. *bulgaricus* belongs to thermophylic lactic acid bacteria and temperatures between 43-46°C are optimal for its growth. Lactic acid bacteria can survive in anaerobic conditions because oxygen is not needed for energy metabolism. They can tolerate aerobic environments as well. A pH modified MRS (pH 4.58) agar and anaerobic incubation at 43°C can be used to

selectively enumerate *L. delbrueckii* subsp. *bulgaricus* from a product (Tharmaraj and Shah, 2003).
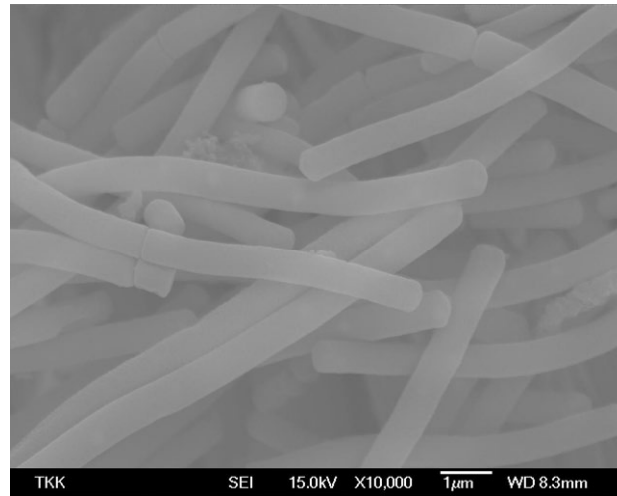

Figure 1. Scanning electron microscopy image of rod-shaped *L. delbrueckii* subsp. *bulgaricus* (courtesy of Kari Lounatmaa).

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the two bacteria required for the production of yoghurt. It plays an essential role in the development of the organoleptic (Ott et al., 1997; Petry et al., 2000), hygienic and perhaps probiotic properties of this food (Hassan and Frank, 2001). **Table 4** gives some of fermented milk products where *L. bulgaricus* is used for production.

Table 4. Dairy products containing *L. delbrueckii* subsp. *bulgaricus* (source Lee and Wong, 1993).

Product	*Starter microorganism*
Yogurt	**L. delbrueckii ssp. bulgaricu**s, *Str. thermophilus*
Bulgarian butter milk	**L. delbrueckii ssp. bulgaricus**
Dahi	**L. delbrueckii ssp. bulgaricus**, *Str. thermophilus, Leu. mesenteroides* ssp. *cremoris*
Kefir	*L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris, L. lactis diacetilactis,* **L. delbrueckii ssp. bulgaricus,** *Str. thermophilus, L. helveticus, L. kefir, Saccharomyces* ssp.
Kumys	*L. acidophilus,* **L. delbrueckii ssp. bulgaricus**, *Saccharomyces lactis, Torula koumiss*

Yogurt has been considered the primary habitat of the species (Davis, 1975) because the bacterium is highly adapted to milk environment (Norbert et al., 1983) and is also able to resist low pH values (Delley and Germont, 2002). However, the millennium long tradition of fermented milk production has propelled the search for plants serving as sources for *L. bulgaricus* isolation. In a historical perspective plant extracts have been added to sheep milk and then heated until a dense milk coagulum is obtained (Markoff, 1925). Several plants have been reported as habitats for *L. bulgaricus*: *Cornus mas*, *Ononis spinosa*, *Berberis vulgaris*, *Paliurus aculeatus*, *Matricaria chamomilla*, *Prunus spinosa* (Girginoff, 1959; Kantardjiev, 1962; Stefanova, 1985; Mychailova et al., 2007). Glucose, fructose, mannose, and sucrose availability on leaf and stem surfaces of these plans are recognized as nutritients that provide optimal growth conditions for the microbial species (Tukey, 1970; Schaffner and Beuchat, 1986; Andrews and Harris, 2000; Mercier and Lindow, 2000; Lee, 2001; Michaylova et al., 2005).

Relative debate exists about whether or not yogurt starter bacteria such as *L. bulgaricus* should be considered probiotics. *In vitro* models and few clinical trials have shown that yogurt bacteria cannot survive in the gastrointestinal tract thus being unable to permanently colonize the gut (Shah and Jelen, 1990; Marteau et al., 1997; del Campo et al., 2005; Garcia-Albiach et al., 2008). In contrast to the intestinal lactobacilli, *L. bulgaricus* does not encode mucin-binding proteins and it is deficient of bile salt hydrolase genes, properties important for survival and activity in the gastrointestinal tract (Klaenhammer et al., 2008). The bacterium has shown no adhesion to human intestinal cells in an *in vitro* system (Elo et al., 1991; Kleeman et al., 1998). However, regular yogurt consumption can be a contributing factor to the establishment and survival of *L. bulgaricus* in upper and lower gastrointestinal tract (Lick et al., 2001; Mater et al., 2005; Elli et al., 2006; Vieira et al., 2008). Additionally, a careful setup of the analytic procedures can improve the reliability of studies regarding the survival of yogurt starters as has been shown by Elli et al. (2006). The adhesion of some strains with known probiotic activity like *Bifidobacterium lactis* Bb12 can be significantly increased in the presence of *L. delbrueckii* subsp. *bulgaricus* when tested *in vitro* (Ouwehand et al., 2000) which, in the context of human microbiota, may highlight synergism among healthy bacteria.

Evidence of plausible probiotic activity of yogurt starter bacteria has been accumulated predominantly from *in vitro* studies. The proposed mechanisms of probiotic activity of *L. bulgaricus* include: 1) antagonism with pathogens by competition for binding sites and/or inhibition of intracellular signaling pathways; 2) stimulation of the mucosal immune system and augmentation of the host defense against pathogenic bacteria and foreign antigens (Nagafuchi et al., 1999).

L. bulgaricus probiotic activity can be ascribed to its ability to produce substances with antimicrobial properties. Lactobacilli are known to inhibit the growth of pathogenic bacteria, possibly by producing inhibitory compounds such as organic acids, hydrogen peroxide and bacteriocins (Jacobsen et al., 1999; Loessner et al., 2003). Bulgarican, lactobulgarican, lactobacillin EG4, lactacin A and B are bacteriocins defined in this species (Reddy et al., 1984; Abdel-Bar et al., 1987; Giraffa et al., 1989; Toba et al., 1991; Nettles and Barefoot, 1993). Evaluating the cultural conditions with respect to bacteriocin

production Balasubramanyam and Varadaraj (1998) have shown that bacteriocin production is strain dependent and can occur from the logarithmic phase through the early stationary phase at the optimal growth temperature, 37 - 45°C and an acidic pH range between 4.0 -5.0 (Reinheimer et al., 1990). Bacteriocins are proteinaceous in nature and stable at 75° C for 30 min. Their inhibition spectrum is narrower than that of antibiotics (McAuliffe et al., 2001; Morency et al., 2001) and their activity is mainly targeted against closely related species. A small (3.6-6 kDa) heat stable bacteriocin containing 29 amino acids from *L. bulgaricus* has shown inhibitory activity against *Listeria monocytogenes*, *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Yersinia enterocolitica* and *Y. pseudotuberculosis* (Miteva et al., 1998). In light of fermented food industry bacteriocin producing strains can be effectively used as food biopreservatives. On the other hand, the deleterious effect of pathogen byproducts on host cells may be diminished in the presence of *L. bulgaricus*. A bioactive component released by a *L. bulgaricus* LDB B-30892 was capable of inhibiting or deactivating the exotoxins released by *C. difficile* thus protecting Caco-2 cells from *C. difficile*-mediated cytotoxicity (Banerjee et al., 2009). Pretreatment with *L. bulgaricus* prior to infection with *E. sakazaki*, known for its ability to stimulate the production of NO leading to apoptosis of IEC-6 cells, was effective in preserving enterocyte integrity both *in vitro* and *in vivo* (Hunter et al., 2009). Furthermore, viable *L. bulgaricus* cells can prevent TLR4 signaling activation and IL-8 production mediated by *H. pylori in vitro*, thus attenuating pathogenic potential of the latter species (Zhou et al., 2008).

However, so far *L. bulgaricus* has not been studied with respect to the inhibition of common oral pathogens.

Immunomodulatory activity has been assessed both in *in vitro* and *in vivo* experiments. The mucosal immune activation is an extremely important characteristic for the selection of probiotic bacteria (Dogi et al., 2008). Yogurt bacteria may potentiate the production and the release of IFN-γ by immunocompetent cells and thereby modulate the host immune response (DeSimone et al., 1986; Makino et al., 2006). *L. bulgaricus* strains can induce cytokine (TNF-a, IL-6, IL-2, and IL-5) secretion in cultured macrophages and T-cells which play a central role in cell-mediated and humoral immunity (Marin et al., 1998). An immunostimulatory oligonucleotide sOL-LB17 found in *L. delbrueckii* supsp. *bulgaricus* strain NIAI B6 could substantially bind to B-cells increasing the number of CD69 positive cells in the Peyer`s patches (Kitazawa et al., 2003).

Consumption of yogurt has been shown to induce measurable health benefits like strengthening of gut barrier function and prevention of intestinal infections; prevention of antibiotic-associated diarrhea and immunomodulation (Pereyra and Lemannier, 1993; Trapp et al., 1993; Meydani and Ha, 2000; Hickson et al., 2007; Zeng et al., 2008). Positive correlation has been observed in the presence of live bacteria when compared with products with heat-killed bacteria (Gilliland and Kim, 1984; Savaiano et al., 1984; Dewit et al., 1988; Lerebours et al., 1989; Van de Water et al., 1999; Rizkalla et al., 2000). Binding of free bile acids by cells of yogurt starter culture bacteria can even be considered as a favorable anti-hypocholesterolemic effect of these species (Pigeon et al., 2002). Moreover, radical scavengers produced in the culture of *L. delbrueckii* subsp. *bulgaricus* 2038 may have a preventive effect on the oxidation of LDL (Terahara et al., 2000).

According to current scientific concepts, Guarner et al. (2005) have proposed yogurt starter cultures to be regarded probiotics if a beneficial physiological effect can be obtained by consumption of the live cultures and the benefit is substantiated appropriately in human studies.

2.5 Issues of safety in the oral health perspective

The growing market of functional foods and widespread use of probiotics has raised the question of their possible health risks. Although lactobacilli and bifidobacteria are ubiquitous in fermented dairy products and possess a GRAS status, there is always the danger that prolonged probiotic intake may cause bacteraemia or endocarditis, transfer antibiotic resistance, and to have detrimental metabolic effects in general (Marteau, 2002; Land et al., 2005; Snydman, 2008, Liong, 2008; Agostoni et al., 2008). However, results from clinical studies have demonstrated that probiotics are well tolerated by various patient groups (Millar et al., 1993; Majamaa and Isolauri, 1997; Pedone et al., 1999; Rosenfeldt et al., 2003; Viljanen et al., 2005) with only a few cases with clinically manifested side effects (Kirjavainen et al., 2003). Hammerman et al. (2006) have concluded that the benefits of probiotics outweigh their potential danger, but yet particular concern must be given to immunocompromised patients and patients with severe conditions (Salminen et al., 2004; Wada et al., 2009). In a randomized, placebo-controlled, cross-over study with human immunodeficiency virus patients probiotic use of *L. rhamnosus* GG was not found to alleviate gastrointestinal symproms or non-infections diarrhea, but was not associated with any adverse effects or infections and therefore can be regarded as safe (Saminen, 2006).

Although probiotics have proven effective against caries pathogens lactobacilli themselves may associate with caries progression. Some strains of *Lactobacillus* spp., together with *S. mutans* have been implicated in caries development (Montalto et al., 2004). The production of organic acids from dietary sugars is a leading factor also in dentin caries progression (Bradshaw and Marsh, 1998). Metabolism and acid production by probiotic lactobacilli anticipated to exert their properties in the mouth should not favor caries induction. Adhesion of two probiotics *L. casei* Shirota and *L. acidophilus* in an artificial caries model have shown inconclusive results about the potential of those species in caries progression; lactobacilli counts were higher in distilled water than in dentin samples under the terms of the study (Lima et al., 2005). A probiotic *L. salivarius* LS 1952R administered to rats in five consecutive days possessed an inherent cariogenic activity after adherence to tooth surface and enhanced cariogenicity of *S. mutans* (Matsumoto et al., 2005). Reproducing oral biofilm model Pham et al. (2009) have observed that *L. salivarius* W24 could establish itself in the biofilm if added simultaneously with the inoculum and it could lower the pH of sucrose-exposed microbiota. These findings indicate that once established in oral microbiota in the presence of sucrose *L. salivarius* W24 might increase the cariogenic potential of the oral microbial community.

Six commercially available lactobacilli, *L. plantarum* 299v, *L. plantarum* 931, *L. rhamnosus* GG, *L. rhamnosus* LB12, *L. paracasei* F19, and *L. reuteri* were assessed for

acid production from various sugars and sugar alcohols (Hedberg et al., 2008). Among them, *L. plantarum* strains had the highest activity fermenting glucose, fructose, lactose, sucrose, maltose, trehalose, and arabinose. Fermentation of glucose, fructose, mannitol, and trehalose by *L. rhamnosus* GG resulted in pH values between 5.2 and 6.8 following 24h incubation. *L. paracasei* and *L. plantarum* displayed very slow fermentation and pH values reaching 5.2 – 6.8 after 72h incubation. The inability of *L. rhamnosus* strains, *L. paracasei* F19 and *L. reuteri* to ferment sucrose adds valuable information about relative safety of these probiotic strains in the caries-prophylactic perspective. Another study addressing sugar fermentation has shown a strain-dependent pH drop and the decrease was the fastest with glucose for all fourteen strains tested, thus highlighting the acidogenic potential of probiotics (Haukioja et al., 2008). Bearing in mind the life long tradition of fermented dairy food consumption without deleterious side effects it can be anticipated that probiotic administration in a milk product is safer than if given in juice without added calcium and phosphorus (Meurman, 2009).

Probiotics – host tissues cross-talk is another aspect of concern. Epithelial cells play essential role in providing innate defense against microbial challenge through the production of antimicrobial molecules, as well as cytokines and chemokines necessary for leukocyte recruitment (Kagnoff and Eckmann, 1997). Studies in gastrointestinal tract have shown very low induction of pro-inflammatory cytokines after probiotic challenge (Ortiz-Andrellucchi et al., 2009; Selvam et al., 2009; Wang et al., 2009). The reaction was markedly strain-dependent (Medina et al., 2007). A significant reduction of TNF-α and IL-8 levels in gingival crevicular fluid has been observed after two weeks intake of a chewing gum with *Lactobacillus reuteri* ATCC 55730 and ATCC PTA 5289 (Twetman et al., 2009). Nonetheless, more specific studies are called for the evaluation of safety with the emergence of new probiotic candidates in the oral cavity.

3 Hypotheses and aims of the study

The main objective of this thesis was to assess *in vitro* the yogurt starter bacterium *L. delbrueckii* subsp. *bulgaricus* for probiotic activity with relevance to the mouth. The final goal would be to evaluate its suitability for oral cavity applications.

Our working hypothesis was that with the daily intake of yogurt starter *L. delbrueckii* subsp. *bulgaricus* some mechanisms in the development of dental caries and periodontal diseases are positively affected.

The specific aims of the studies were to assess:

1. the antimicrobial activity of *L. delbrueckii* subsp. *bulgaricus* strains against various oral pathogens (oral streptococci, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Fusobacterium nucleatum*);

2. the ability of dairy *L. delbrueckii* subsp. *bulgaricus* strains to adhere to saliva-coated surfaces and to evaluate whether this species might affect the adhesion of oral streptococci *in vitro*;

3. the proteolytic activity on human gelatinases of *L. delbrueckii* subsp. *bulgaricus* strains isolated from yogurt, thus addressing the issue of safety which is a prerequisite for further research on the role of this species on oral health;

4. the epithelial cell response after stimulation with *L. delbrueckii* subsp. *bulgaricus* and the interference of the species on cytokine response provoked by *P. gingivalis*.

4 Materials and methods

4.1 Bacterial strains and culture conditions

4.1.1 Lactic acid bacteria

Strains of lactic acid bacteria used in the studies and their culture conditions are listed in *Table 5*. *L. delbrueckii* subsp. *bulgaricus* strains were kindly provided by LB Lactis (Scientific-Applied Laboratory for Starter Cultures and Probiotic Products, Plovdiv, Bulgaria) culture collection in milk medium. They were subcultured in de Man, Rogosa and Sharpe (MRS) broth at pH 6.4 at 37°C in 5% CO_2 atmosphere for 24 h. The lactobacilli were verified by Gram staining and carbohydrate fermentation patterns (API 50 CHL, BioMerieux®, Lyon, France). The strains were maintained as frozen stock in 10% skim milk at -70°C between different studies.

Table 5. Lactic acid bacteria used in the studies and their culture conditions

Strain	Origin	Growth medium	Atmosphere	Incubation time	Article
L. bulgaricus LBL-12	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III**
L. bulgaricus LBL-22	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III**
L. bulgaricus LBL-6	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III**
L. bulgaricus LBL-83	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III, IV**

L. bulgaricus LBL-9	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III**
L. bulgaricus LBL-11	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III, IV**
L. bulgaricus LBL-23	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, III**
L. bulgaricus LBL-10	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, IV**
L. bulgaricus LBL-13	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II**
L. bulgaricus LBL-42	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**I, II, IV**
L. bulgaricus LBL-3	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**II, IV**
L. bulgaricus LBL-20	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**II, IV**
L. bulgaricus LBL-39	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**II, IV**
L. bulgaricus LBL-43	Laboratory collection, LB	MRS	5% CO_2	O/N	**II**

Strain	Origin	Growth medium	Atmosphere	Incubation time	Article
	Lactis, Bulgaria				
L. bulgaricus LBL-81	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**II, IV**
L. bulgaricus LBL-80	Laboratory collection, LB Lactis, Bulgaria	MRS	5% CO_2	O/N	**IV**
L. bulgaricus ATCC 11842	Valio Ltd., Helsinki, Finland	MRS	5% CO_2	O/N	**I, III**
L rhamnosus GG ATCC 53103	Valio Ltd., Helsinki, Finland	MRS	5% CO_2	O/N	**I, II, IV**
L. rhamnosus Lc705	Valio Ltd., Helsinki, Finland	MRS	5% CO_2	O/N	**I**
L. casei 921 ATCC 344	Valio Ltd., Helsinki, Finland	MRS	5% CO_2	O/N	**I**
L. casei Shirota	Yakult, Tokyo, Japan	MRS	5% CO_2	O/N	**I**

Abbreviations: O/N: over-night (16-18h).

4.1.2 Oral bacteria

Oral bacteria used in studies I and II, their origin and growth media are listed in *Table 6*.

Table 6. Oral bacteria used in the studies.

Strain	Origin	Growth medium	Atmosphere	Incubation time	Article
S. constellatus ATCC 27823	ATCC	BHI	5% CO_2	24 h	**I**
S. intermedius ATCC	ATCC	BHI	5% CO_2	24 h	**I**

39

27335					
S. mitis ATCC 33399	ATCC	BHI	5% CO_2	24 h	**I**
S. mutans ATCC 25175	ATCC	BHI	5% CO_2	24 h	**I**
S. oralis ATCC 35037	ATCC	BHI	5% CO_2	24 h	**I**
S. sobrinus ATCC 33478	ATCC	BHI	5% CO_2	24 h	**I**
S. salivarius ATCC 13419	ATCC	BHI	5% CO_2	24 h	**I**
S. anginosus ATCC 33397	ATCC	BHI	5% CO_2	24 h	**I**
A. actinomycetemcomitans ATCC 29523	ATCC	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans ATCC 43718	ATCC	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans ATCC 33384	ATCC	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans ATCC 37399	ATCC	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans ATCC 381	ATCC	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans F1000	Clinical isolate	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans F 296	Clinical isolate	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans F 731	Clinical isolate	TS	5% CO_2	24 h	**I**
A. actinomycetemcomitans F 982	Clinical isolate	TS	5% CO_2	24 h	**I**
F. nucleatum ATCC	ATCC	Brucella agar	Anaerobic	72 h	**I**

25586					
P. gingivalis Q 710	Clinical isolate	Brucella agar	Anaerobic	72 h	**I**
P. gingivalis Q 282	Clinical isolate	Brucella agar	Anaerobic	72 h	**I**
P. gingivalis Q 118	Clinical isolate	Brucella agar	Anaerobic	72 h	**I**
P. gingivalis Q 677	Clinical isolate	Brucella agar	Anaerobic	72 h	**I**

4.1.3 Cell cultures

Human mucosal keratinocyte cell line Tuija was used in **study IV**. This cell line has been obtained from surgical gingival biopsies and cultured in serum-free low calcium Keratinocyte Basal Medium (KBM) (Salo et al., 1991) and thereafter underwent spontaneous immortalization due to transfection with human papilloma virus (Pirisi et al., 1988). Tuija cells were grown in KGM 2 supplemented with 0.15mM $CaCl_2$, 2 ml BPE-15, 0.125 ng ml^{-1} epidermal growth factor, 5 µg ml^{-1} insulin, 0.33µg ml^{-1} hydrocortisone, 10 µg ml^{-1} transferrin, and 0.39 µg ml^{-1} epinephrine, at 37°C and passages between 35-45 were used in the study. For separate experiments, cells were seeded at a concentration of 2 x 10^{5} cells ml^{-1} in 24-well tissue culture plates (Nunc, Roskilde, Denmark), and grown at 37°C in 5% CO_2 until forming a monolayer with approximately 85% confluence.

4.2 Methods

Methods used in the separate studies are listed in *Table 7* and described in detail in the original articles.

Table 7. Methods used in studies I – IV.

Method	Described and used in
Agar-overlay inhibitory assay	**I**
Streak-line inhibitory test	**I**
Quantitative assessment of adhesion of radiolabeled bacteria to saliva-coated surfaces	**II**

Western blotting	**III**
SDS-PAGE	**III**
ELISA	**IV**

4.3 Study design

4.3.1 Antimicrobial activity against various oral pathogens (I)

To study the inhibitory activity of lactobacilli against renowned and putative oral pathogens (strains listed in Table 6) the agar overlay and streak line inhibitory assays were used dependent on the target bacteria.

Agar overlay method as described by Kakessy and Piguet (1970) was used to determine the inhibitory activity of lactobacilli against oral streptococci and *A. actinomycetemcomitans*. The inhibition zones were measured after incubation for 24 h at 37°C in 5% CO_2. To measure the inhibitory activity the following formula (Coeuret et al., 2004) was used:

$$\text{Effective inhibition ratio (EIR)} = \frac{\text{ID} - \text{CD}}{\text{CD}}$$

Where, ID is the diameter of the inhibition halo, CD is the diameter of the colony. Scores below 0.5 are defined as slight inhibitory activity, between 0.5 and 1.5 as intermediate, and scores above 1.5 represent strong inhibition.

Streak-line inhibitory activity test was performed according to Annuk et al. (2003) to study the inhibitory activity of lactobacilli against *P. gingivalis* and *F. nucleatum* strains. After 72 h incubation the width of the zone of inhibition (mm) extending from the target bacteria to the lactobacilli streak line was measured (Mikelsaar et al., 1987).

4.3.2 Adhesion to saliva-coated surfaces in vitro (II)

For adhesion studies the bacteria (listed in Table 5) were radiolabeled by growing the cells in appropriate broth supplemented with 10 μl/ml of [methyl-1,2-3H]thymidine, 122 Ci/mmol (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) as previously described (Fernandez et al., 2003).

Unstimulated whole saliva was collected from five healthy individuals who were instructed not to eat, drink, smoke, or use chewing gum for an hour before the saliva collection. Informed consent was obtained before the collection began. The saliva was collected into chilled tubes on ice and clarified by centrifugation (14,000 g for 20 min at 4°C). The pooled samples were divided into aliquots and frozen at -20°C before the adhesion assays.

4.3.2.1 Adhesion to sHA beads

Spheroid HA beads (Macro-Prep Ceramic Hydroxyapatite TYPE II 80 µm, Bio-Rad Laboratories, Hercules, CA) were equilibrated for 2 h in buffered KCl (0.05 m KCl containing 1 mm KH_2PO_4, 1mm $CaCl_2$ and 0.1 mm $MgCl_2$ at pH 6.5). 100 µl saliva was added per well and the mixture was incubated for 1 h at 37°C. After three washings with buffered KCl (200 µl/well) 100 µl radioactive bacterial suspension was added to each well and incubated with shaking (50 r.p.m.) for 1 h. The radioactivity was measured by liquid scintillation counter (Winspectral 1414, Wallac, Turku, Finland). The adhesion ratio (%) of bacteria was calculated by comparing the radioactivity of the adhered bacteria to the radioactivity of the added bacteria.

4.3.2.2 Adhesion to saliva-coated microtiter plates

Adhesion to human saliva was assessed according to the method studying adhesion to intestinal mucus as described earlier by Ouwehand et al., (2001). Saliva was immobilized passively overnight at 4°C in 96-well polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark; 100 µl/well). Bacterial suspensions were added (100 µl/well) and bacteria were allowed to adhere at 37°C for 1 h. Lactobacilli pretreated with lysozyme (0.05 mg/ml in phosphate-buffered saline (PBS), pH 6.2) were assessed for their ability to adhere to saliva.

4.3.2.3 Adhesion to solvents

Microbial adhesion to n-hexadecane was measured according to the method of Rosenberg et al. (1980). A detailed description of the method is given in **study III**.

4.3.2.4 Effect of lactobacilli pretreatment on streptococcal adhesion in vitro

To study the effect on adhesion of *S. sanguinis* ATCC 10556 after *Lactobacillus* pretreatment of saliva-coated MaxiSorp plates, non-radiolabeled *L. delbrueckii* subsp. *bulgaricus* strains were allowed to adhere to immobilized saliva for 1 h at 37°C. After two washes with HEPES–Hanks' buffer, 100 µl streptococcal suspension was added per well and incubated for 1 h at 37°C and the adhesion experiment was performed as already described.

4.3.3 Proteolytic activity on human gelatinases (III)

The proteolytic activity of different *L. bulgaricus* strains (listed in Table 5) on human progelatinase B (pro-MMP-9) was evaluated based on a protocol used for assessing the gelatinolytic activity of defined oral pathogens (Grayson et al., 2003).

Lactobacilli were grown in de Man, Rogosa and Sharpe broth at pH 6.4 (MRS broth, LAB MTM, IDG Ltd., Lankashire, UK) at 37°C in 5% CO_2 atmosphere. Cells were harvested by centrifugation at 5 000 g for 20 min, and the supernatants were dialyzed against distilled water for 2 h at 4°C. Harvested cells were washed twice with PBS, pH 7.4, suspended in 1 mL of PBS. Prior to use they were sonicated on ice to disrupt the cells. Both cell fractions and the supernatant fractions were used in this study.

The presence of gelatinolytic proteases was assayed with the use of an enzymography in 0.75-mm-thick 11% SDS-PAGE gels impregnated with 1 mg mL^{-1} gelatin, as described in **Study III**. White zones of lysis indicating gelatine degradation were revealed by staining with 1% Coomassie Brilliant Blue.

The molecular forms of MMP-9 were detected by a modified (Sorsa et al., 1997) ECL Western blotting kit according to protocol recommended by the manufacturer (GE Healthcare, Amersham, UK).

To determine the inhibitory effect of different synthetic MMP inhibitors on *L. bulgaricus* proteases, Ilomastat (Chemicon International Inc., CA, USA), EDTA (Merck, KGaA, Dramstadt, Germany), CMT3, CMT308 (Collagenex Inc., Newton, PA, USA), CTT1 (Koivunen et al., 1997) and a serine protease inhibitor, Pefabloc (Boehringer Mannheim GmbH, Manheim, Germany), were employed in this study. The MDPF-zymography was assayed as previously to detect the residual gelatinolytic activity.

4.3.4 Epithelial cell response to *L. delbrueckii* subsp. *bulgaricus*

4.3.4.1 Induction of IL-8 and TNF-α secretion

Lactobacilli in KGM 2 or culture supernatants (1 ml) were added to the epithelial cell monolayers and incubated for 6h and 24h at 37°C in 5% CO_2. KGM 2 alone served as a negative control. At each time point 500µl of the cell culture medium was removed and centrifuged to obtain debris-free supernatant. Collected supernatants were stored at -20°C until ELISA assessment.

4.3.4.2 Epithelial cell response to P. gingivalis after lactobacilli pretreatment

After 24h incubation with lactobacilli the epithelial cells were washed twice with PBS and *P. gingivalis* in KGM 2 was added to a volume of 1 ml to each well. The epithelial cell monolayers were then cultured at 37°C in 5% CO_2. After 1, 2 and 24h 500µl supernatants were collected for ELISA analyses.

4.4 Statistical analyses

All experiments were run at least in duplicate and scores are presented as means ± SEM. Differences were considered significant when P<0.05. Appropriate parametric and non-parametric tests employed are described in detail in **studies I-IV**.

5 Results and discussion

The present series of studies addressed several key aspects of probiotic activity to be assessed with respect to possible oral cavity applications.

5.1 Inhibition of oral pathogens (Study I)

Thirty lactobacilli strains, 11 of which were *L. bulgaricus*, were assessed for their inhibitory activity against 23 strains of oral pathogens.

   A. actinomycetemcomitans strains were the most susceptible to the inhibitory activity of the lactobacilli tested. The inhibition varied significantly from slight to strong ($p < 0.05$). Four *L. bulgaricus* strains, namely *L. bulgaricus* LBL-9, *L. bulgaricus* LBL-11, *L. bulgaricus* LBL-23, and *L. bulgaricus* LBL-83, showed the most pronounced inhibitory activity among all strains tested (EIR>2). No difference was observed in growth inhibition between the clinical isolates of *A. actinomycetemcomitans* vs. commercial strains. **Table 8** presents the inhibitory activity of *L. bulgaricus* strains against *A. actinomycetemcomitans*. Our results are in agreement with the results by Koll-Klais et al., (2005) who reported that homo-fermentative lactobacilli expressed significant antimicrobial activity against periodontal pathogens. *L. bulgaricus* is an obligate homofermentative and most of the strains strongly inhibited *A. actinomycetemcomitans*.

Table 8. Inhibition of clinical and commercial isolates of *A. actinomycetemcomitans* by *L. delbrueckii* subsp. *bulgaricus* strains tested.

Strain	*Aggregatibacter actinomycetemcomitans*									
	ATTC 29523	ATCC 43718	ATCC 33384	ATCC 37399	ATCC 381	F 1000	F 296	F 731	F 982	Mean ±SD
L. bulgaricus 365	**2.4**	**1.4**	**1.2**	**1.9**	**1.3**	**1.3**	**1.8**	**1.8**	**1.5**	**1.6±0.3**
L. bulgaricus LBL-23	**2.2**	**2.8**	**3.2**	**2.4**	**1.4**	**1.5**	**2**	**1.5**	**2.1**	**2.1± 0.5**
L. bulgaricus LBL-12	**2.9**	**0.8**	**0.8**	**1.2**	**1.1**	**0.9**	**1.4**	**1.1**	**1.3**	**1.6± 1.6**
L.	**0**	**0**	**0**	**0**	**0.5**	**0**	**1.3**	**0.1**	**0.6**	**0.3± 0.4**

bulgaricus LBL-22										
L. bulgaricus LBL-6	3.2	0	0	0	0	0.3	0	0.6	1.1	0.6±0.05
L. bulgaricus LBL-10	2.2	2.3	2.8	2.5	0.9	2.5	1.1	1.9	2.9	2.1±0.7
L. bulgaricus LBL-13	0.5	3	2.3	1.7	0	1	1.3	1.2	0.7	1.3±0.9
L. bulgaricus LBL-83	4	4.5	3.8	2.1	1.3	1.1	1.1	2.5	2.2	2.5±1.3
L. bulgaricus LBL-42	1.6	4.2	2.2	2.1	1.7	1.6	2.1	1.2	2	2± 0.8
L. bulgaricus LBL-9	2.2	3.8	3.5	3.3	2	1.3	2.4	1.3	2.2	2.4±0.9
L. bulgaricus LBL-11	3.5	2.8	3.7	4.4	1.5	1.6	1.8	1.8	1.6	2.5±1.1

Oral streptococci showed various patterns of susceptibility to lactobacilli. In general the average inhibitory activity of lactobacilli against streptococcal species was low (EIR = 0.5). Among the lactobacilli tested only strains of *L. bulgaricus* inhibited the growth of oral streptococci. The pattern of inhibitory activity of *L. bulgaricus* strains against streptococcal species is given in *Figure 2*. In the present study *S. mutans* was strongly inhibited by single *L. bulgaricus* strains (*L. bulgaricus* LBL-23 being the strongest). Strahinic et al. (2007) and Koll-Klais et al. (2005) have shown that *S. mutans* is susceptible to growth inhibition by various oral lactobacilli. Considering the common use of yogurt in the diet of many populations dairy strains may indeed affect the composition of oral biofilm formation already in early childhood if the dairy starter bacteria interact with commensal microflora, thus altering its cariogenic potential.
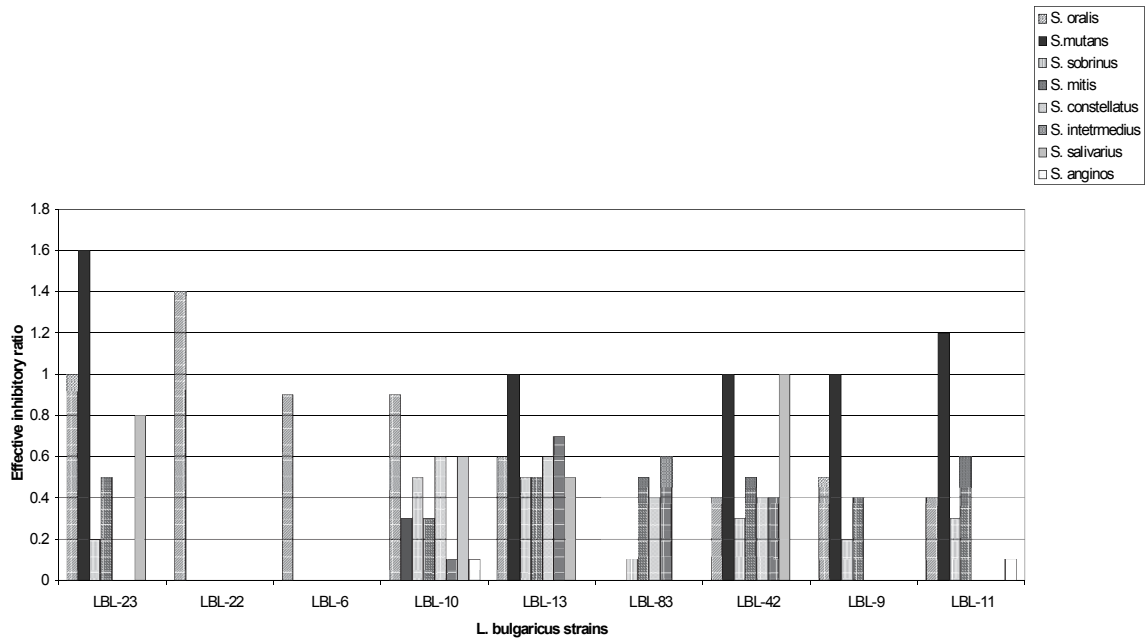
Figure 2. Effective inhibition ratio against oral streptococci by *L. bulgaricus.*

The mechanisms by which *L. bulgaricus* strains inhibited oral pathogen growth are not fully understood. Lactobacilli may exert their antibacterial activity through the production of organic acids (lactic and acetic acid) and other metabolites such as hydrogen peroxide and diacetyl, or specific bactericidal or bacteriostatic peptides and proteins (De Vuyst et al., 1994). We observed that when lactobacilli were grown on MRS agar with normal glucose content, instead of 0.2% glucose agar, growth inhibition of both the streptococci and *A. actinomycetemcomitans* was more pronounced. Koll-Klais et al. (2005) reported the same which indicates that the availability of substrate for fermentation seems to be one of the essential factors for the antimicrobial activity. We have found that culture supernatants of lactobacilli possessed no antimicrobial activity against streptococci and *A. actinomycetemcomitans* when using the well-diffusion or paper-disk assays according to Drago et al., (1997). Hence, the inhibitory mechanisms may be cell bound functions.

Streak line inhibition test used to study the effect of lactobacilli against *P. gingivalis* and *F. nucleatum* showed low susceptibility of these bacteria. There was no inhibitory activity observed among *L. bulgaricus* strains against either *P. gingivalis* or *F. nucleatum.*

The inhibitory pattern varied distinctly between the lactobacilli tested. However, there was no single *Lactobacillus* strain to demonstrate growth inhibition against all four oral pathogen species used. Thus, no single *Lactobacillus* species can be used in combating oral pathogens in broader sense and several species need to be considered when selecting "oral probiotics".

48

5.2 *L. bulgaricus* adhesion to saliva-coated surfaces (Study II)

Adhesion of bacteria to host surfaces is regarded of major importance in contributing to permanent, or even transient, establishment of probiotic species in any environmental niche. In the present study we focused on the bacterial adhesion to human saliva that is the main fluid overlying oral surfaces. Presumably probiotic bacteria that express good binding ability to salivary pellicle may also be able to colonize the oral cavity.

Saliva-coated HA beads have been commonly used as an *in vitro* model to quantitatively study adhesion of radiolabeled bacteria because the surface properties are similar to those of tooth enamel (Gibbons et al., 1982). Adhesion to sHA varied between 1 to 17%, and *L. bulgaricus* LBL-39 exhibiting values comparable to that of the reference strains *S. sangunis*. *S. sanguinis* is the first colonizer on tooth surfaces *in vivo* and its ability to adhere to sHA make it a suitable model for dental adhesion studies.

Generally, the adhesion of most *L. delbrueckii* subsp. *bulgaricus* strains to sHA was low (<5%) under the present experimental conditions. The adhesion to saliva-coated Maxisorp plates ranged between 3 and 22%, with LBL-39 exhibiting the strongest ability to adhere.

A significant increase in the adhesive properties was observed when the strains were pretreated with lysozyme (P < 0.05); results are shown in *Figure 3.*
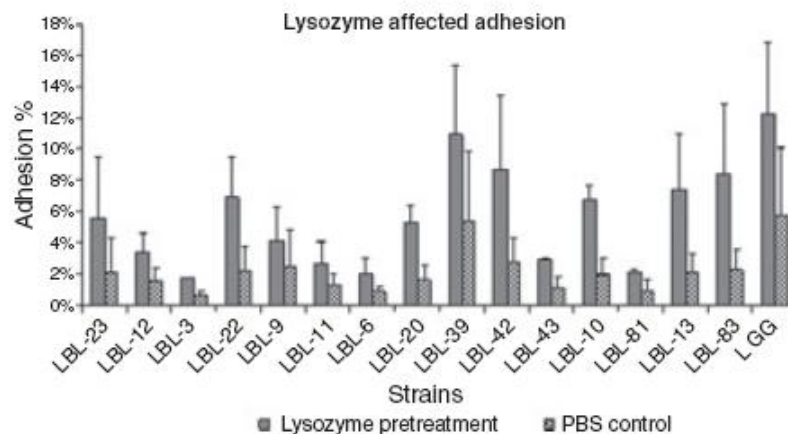

Figure 3. Adhesion of *L. bulgaricus* to saliva-coated Maxisorp plates after lysozyme pretreatment.

Tellefson and Germaine (1986) have found that lysozyme promoted the adherence of some oral streptococci (*S. sanguinis*) to sHA. The role of lysozyme pretreatment on probiotic properties has recently been also addressed as a factor improving the immunostimulatory effect of probiotic species (Bu et al., 2006).

Cell surface hydrophobicity has been considered a valuable reference when evaluating the adhesive properties of microorganisms. High hydrophobicity correlated with marked adhesion (Wadström et al., 1987). The assessment of cell surface hydrophobicity might be used as a test for studying adhesive properties of bacteria because this characteristic has been reported to objectively reflect microbial adhesion (Ellepola et al., 2001; Wadström et

al., 1987). By measuring adhesion to n-hexadecane we observed that the strains investigated showed various patterns of interaction with the organic solvent, as shown in *Figure 4*. The *Lactobacillus* strain LBL-39 which had shown the most pronounced adhesive properties to saliva-coated surfaces again displayed the strongest adhesive potential.
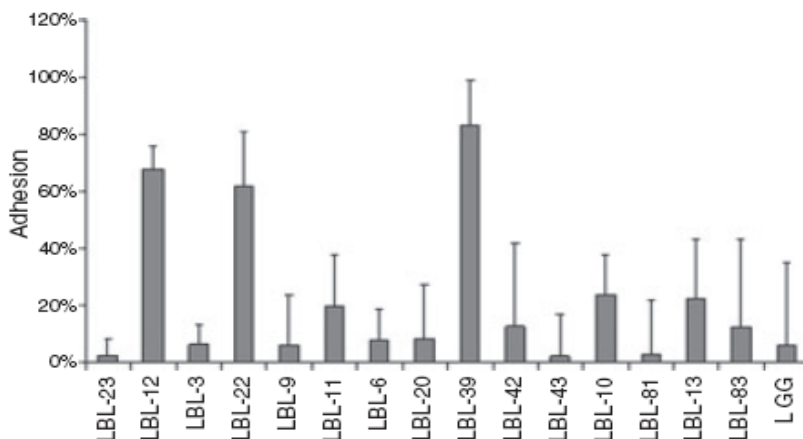

Figure 4. Adhesion of *L. bulgaricus* to n-hexadecane.

As *S. sanguinis* and lactobacilli were able to adhere to saliva-coated surfaces we hypothesized that these two species may compete when present together. However, the adhesion of *S. sanguinis* ATCC 10556 was not significantly affected by the pretreatment of the wells with any of the *Lactobacillus* strains, as shown in *Figure 5.*
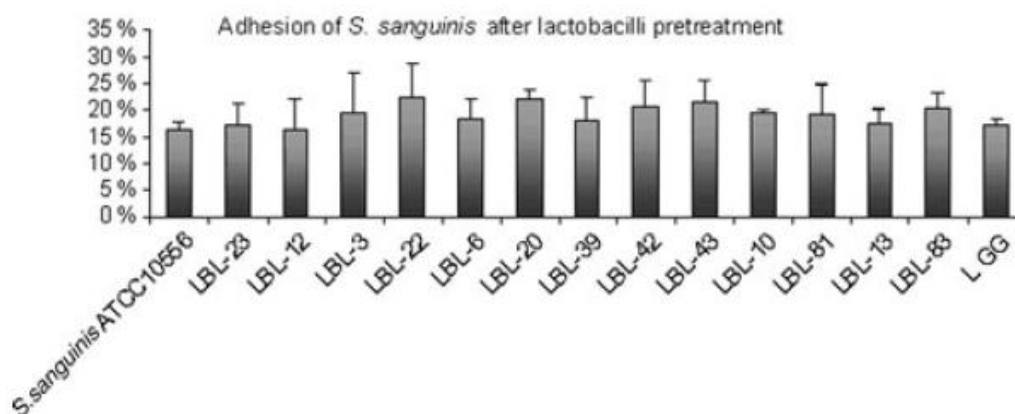
Figure 5. Adhesion of *S. sanguinis* ATCC 10556 to saliva-coated microtiter plates after pretreatment with the lactobacilli studied.

The competitive inhibition for bacterial adhesion sites has been considered as a favorable mechanism for probiotic action (Fernandez et al., 2003). Despite the fact that lactobacilli

adhered to various extents to the immobilized saliva they were not able to affect the adhesion of the target microorganism tested. It could therefore be concluded that the salivary receptors are different for dairy strains and *S. sanguinis* and that pretreatment with lactobacilli does not block streptococcal adhesion by steric hindrance. Similar results were observed for other probiotic species that also lacked the capacity to change the adhesive potential of several skin pathogens (Ouwehand et al., 2003).

Issues of safety demand substantial consideration and *in vitro* tests are critical when assessing the mechanisms of probiotic effect with no hazards being imposed on the host by the use of living microorganisms in therapy. Safety issues of lactobacilli have been studied by evaluating adhesion to main constituents of extracellular matrix: collagen type IV and fibrinogen; binding to intestinal mucus; induction of respiratory burst in peripheral blood monocytes and resistance to serum-mediated killing (Vesterlund et al., 2007). There were no studies addressing the issues of safety related to screening of putative probiotic species with the scope of application in the oral cavity. A favorable metabolic activity and harmless host-bacteria interactions that pose no risk to oral health of the individual must be considered when putative probiotics are administered in the mouth. *L. delbrueckii* subsp. *bulgaricus* that is essential in yogurt production exerts high hydrolysing activities towards substrates containing proline, alanylprolyl–p–nitroanilide and prolyl–p–nitroanilide (Sasaki et al., 1995). It is known that *L. delbrueckii* subsp. *bulgaricus* possesses a complex proteolytic system essential for rapid growth in protein-rich media (Atlan et al., 1994) and the hydrolysis of milk caseins by means of a cell-wall proteinases has been extensively studied (Smid et al., 1991; Laloi et al., 1991). A cell-envelope-associated aminopeptidase characterized as metallo-enzyme with a broad specificity has been purified from the cell wall of *L. bulgaricus*, *L. lactis*, and *L. helveticus* (Atlan et al., 1989; Blanc et al., 1993).

5.3 Proteolytic activity on human progelatinase B (proMMP-9) (Study III)

In the present study we applied a method evaluating the effect of probiotic candidates on the activation of matrixmetalloproteinases (MMPs), the enzymes responsible for extracellular matrix degradation and remodeling. Elevated levels of salivary MMPs have been associated with metabolic activity of various oral pathogens (Ding et al., 1997; Mäntylä et al., 2003; Söder et al., 2006). Thus, the capacity of some microbial species to convert extracellular matrix enzymes into their active forms might be considered an inherent virulence factor.

5.3.1 Gelatinolytic activity

Gelatin zymography with labeled substrate enables the detection of type I and type IV collagenolytic activity. The gelatinolytic activity of all strains tested was very low compared with positive human saliva controls. Degradation of gelatin was not detected

after an 18 h incubation period. However, the prolonged 7-day incubation time yielded molecular weight bands at the area of 106 kDa and around 150 kDa (**Figure 6).** There was no significant difference in the gelatinolytic activity when the different pH values of the buffers were used. Supernatant samples, although showing only weak proteolytic activity, were more potent in degrading gelatin than the cell fraction samples which yielded no visible bands on the UV light picture. No difference was observed among the strains in the degradation of gelatin.
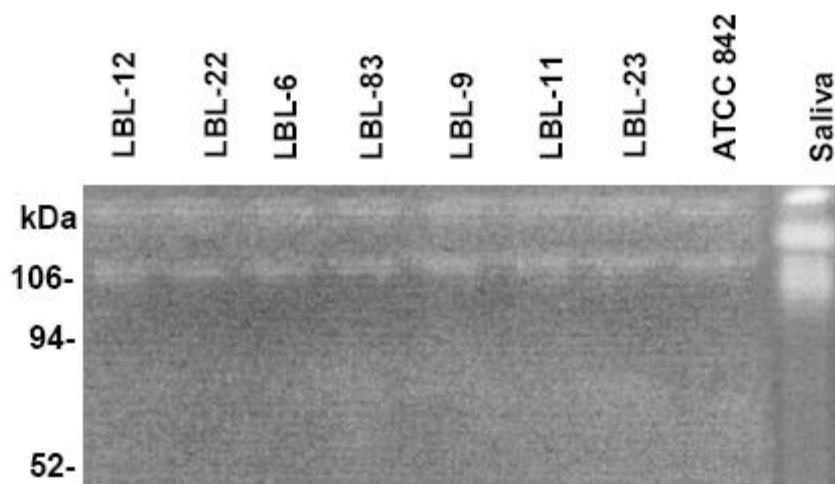
Figure 6. Gelatinolytic activity of *L. delbrueckii* subsp. *bulgaricus* supernatants. All strains show similar bands of activity after a 7-day incubation period.

Considering the attachment to oral mucosa, it is essential that the microorganism is not harmful to mucosal cells and extracellular matrix and basement membrane components. A damaged or disintegrated oral epithelium facilitates a microbial invasion, providing appropriate environment for further bacterial growth. Most bacterial proteinases, however, have weak degrading activity against collagen (Okamoto et al., 2004). Once activated human collagenolytic MMPs might provide suitable substrate for further activity of human gelatinases or other bacterial proteinases. The test strains investigated in our study demonstrated very low gelatinolytic activity even after the longest incubation period, which validates their relative safety as probiotic candidates.

5.3.2 Activation of proMMP-9

ProMMP-9 was incubated for three different time periods with supernatants and cell fraction suspensions of the *L. bulgaricus* strains. The conversion of proMMP-9 into its

active form was not detected after 24h of incubation as shown by Western blotting with the anti-MMP-9 antibodies (***Figure*** 7A and 7B).
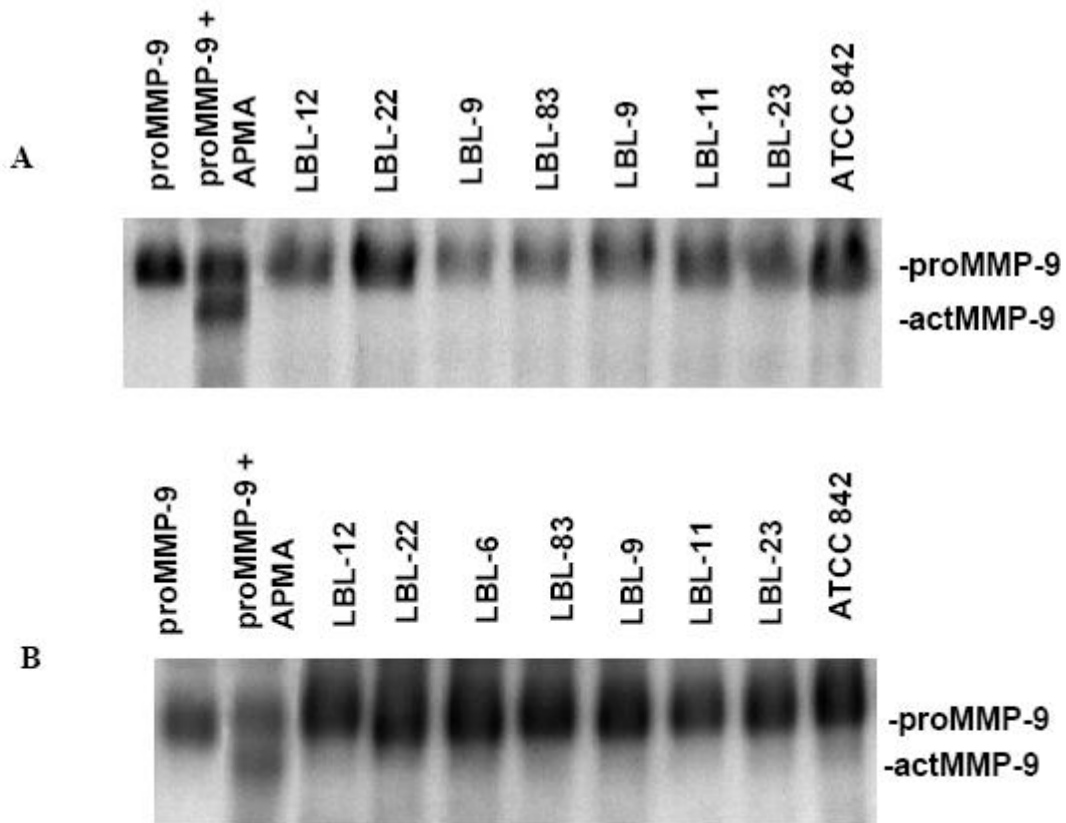

Figure 7. *L. delbrueckii* subsp. *bulgaricus* strains are ineffective in converting proMMP-9 to its active form as shown on Western blotting images. A. Supernatant fractions; B. Cell fractions.

MMPs are expressed at low levels in the absence of inflammation, wound healing or other pathological processes (Woessner, 1991).

MMP-9 and other endogenous proteinases hydrolyze and degrade the fragments of denatured collagens, for example gelatin, into smaller fragments. It has been shown in many studies that MMP-9 is a specific marker for periodontal destruction (Ejeil et al., 2003) and elevated levels of this enzyme are related to the severity of periodontal breakdown. Referred to as type IV collagenase MMP-9 is particularly implicated in the degradation of the basement membrane (Reynolds and Meikle, 1997). The proteolysis of the ECM seems to play an important role in initiating the progression of the inflammatory process, and thus conversion of proMMPs into their active forms is a crucial step here, facilitating bacterial adhesion and infection. Studies on the activation of human MMPs have shown that some bacterial species with clear pathogenic potential are capable of activation of MMPs. For example, *Vibrio* proteinase and *Pseudomonas* elastase have shown stronger activation of pro-MMP-9 than did APMA (Okamoto et al., 1997). Furthermore, pro-MMPs can be activated by a variety of mechanisms that include proteinases such as plasmin; thiol-oxidizing agents, e.g., $HgCl_2$ and N-ethylmaleimide;

low pH; and heat treatment (Vise and Nagase, 2003). MMPs are secreted as proenzymes and their activity is low in intact normal tissues but could undergo activation by a broad range of stimuli (Sorsa et al., 1997; Johnson et al., 1998; Potempa et al., 2000; Okamoto et al., 2004). A key event in the activation of proMMPs is the removal of the propeptide domain in their structure that usually consists of ca. 80 amino acid residues (Nagase et al., 1990). *Lactobacillus bulgaricus* strains in our study were incapable in converting the proMMP-9 to the 60–80 kDa forms considered active and did not show any activity at the region of the molecular mass consistent with protease IV.

5.3.2 Proteolytic activity of *L. bulgaricus* strains in the presence of synthetic MMP inhibitors

To investigate if the synthetic inhibitors of MMPs affect the gelatinolytic activity of bacterial proteases, the *L. bulgaricus* strains tested were incubated with five different synthetic MMP inhibitors and Pefabloc. No significant changes in gelatinolytic activity were observed on Coomassie Brilliant Bule stained gels. Synthetic MMP inhibitors and Pefabloc did not affect the proteolytic activity of the supernatants or the cell fraction suspensions of the *L. bulgaricus* strains investigated.

The administration of synthetic inhibitors of MMPs is considered a therapeutic approach in the treatment of different pathological conditions in which elevated levels of MMPs are regarded as key factors in inflammation and tissue breakdown. The preserved and unaffected proteolytic activity of the test strains after addition of different synthetic MMP inhibitors and Pefabloc in the test system might additionally benefit the anticipated probiotic effect of those microorganisms. Consequently, a simultaneous administration of potential probiotics and inhibitors of MMPs should not be regarded contradictory when potential new treatment modes for infectious diseases are being considered.

5.4 Epithelial cell – lactobacilli interactions (Study IV)

In vitro experiments as conducted here are the first step in the evaluation of safety aspects when the oral cavity is exposed to high numbers of lactobacilli. The integrity of the epithelial lining of the oral cavity is part of the innate defense and serves as an effective barrier against various microorganisms.

The ability of oral epithelial cells to secrete proinflammatory cytokines, IL-8 and TNF-α, in response to five different lactobacilli species at two different concentrations, 10^6 CFU ml^{-1} and 10^9 CFU ml^{-1}, was examined. The viability of cultured epithelial cells remained above 85% during the whole set of experiments.

In the present study the strongest induction of IL-8 secretion was observed with live bacterial samples at the higher concentration (10^9 CFU ml^{-1}) compared to heat killed bacteria ($p < 0.05$). The increased levels of IL-8 were concentration dependent. Heat killed bacterial samples at concentration of 10^6 CFU ml^{-1} were stronger inducers of IL-8 than heat killed bacteria at 10^9 CFU ml^{-1} ($p < 0.05$). One strain, namely *L. bulgaricus* LB-86,

induced significantly lower secretion of IL-8 compared with *A. actinomycetemcomitans* positive controls (p < 0.05). The remaining strains within this group showed IL-8 values similar to those measured for *A. actinomycetemcomitans* at the 6h incubation-point. Heat killed *L. bulgaricus* LB-39, *L. bulgaricus* LB-3, *L. bulgaricus* LB-11, *L. bulgaricus* LB-42, *L. bulgaricus* LB-86 induced significantly higher levels of IL-8 compared to their live counterparts 6h after co-culturing with the epithelial cells. *Figure 8* shows the dynamics of IL-8 secretion within 24h of co-culturing of lactobacilli with the epithelial cells. Bacterial culture supernatants of all the strains tested led to undetectable levels of IL-8 in the culture medium. Furthermore, the addition of *P. gingivalis* to the epithelial cells pretreated with lactobacilli showed an almost immediate disappearance of any detectable levels of IL-8 in culture medium.
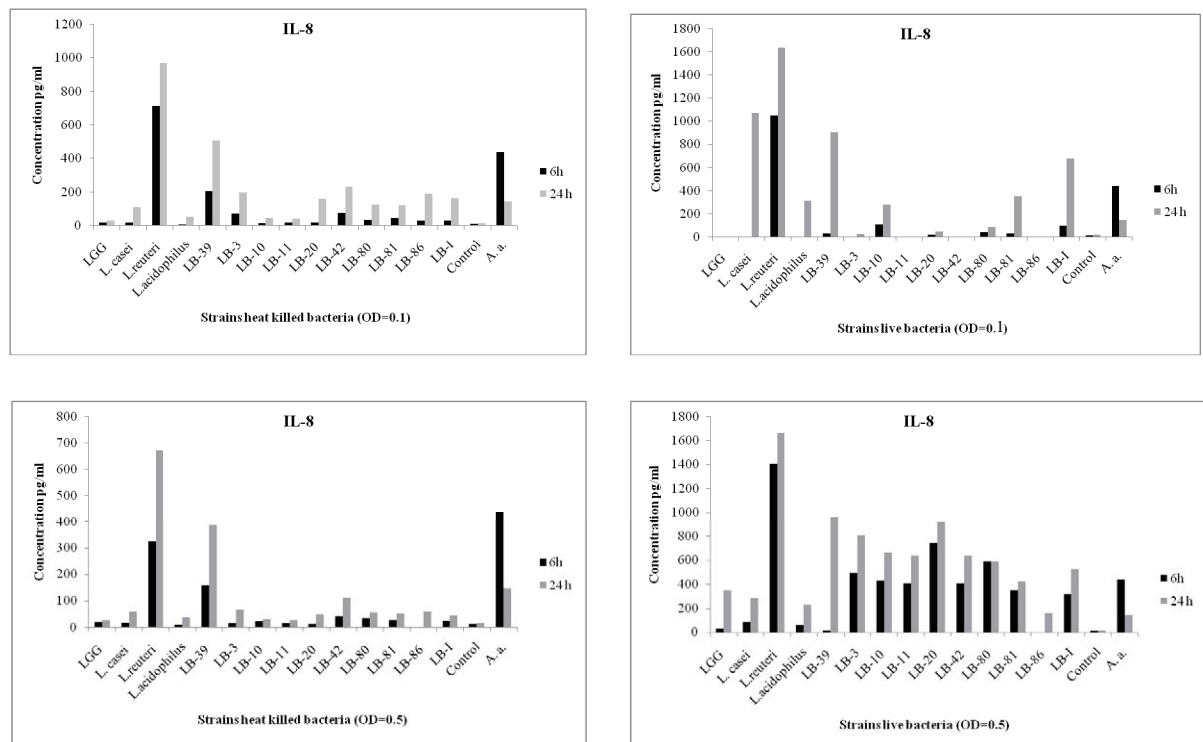

Figure 8. Levels of IL-8 secreted after co-culturing of oral epithelial cells with heat killed and live lactobacilli strains at two different concentrations (OD = 0.1 and OD = 0.5, corresponding to 10^6 and 10^9 CFU ml^{-1}). Supernatants were collected at 6 and 24h.

After co-culturing with lactobacilli the epithelial cells responded with different concentrations of TNF-α secreted in the culture medium. Generally, the concentration of TNF-α was low in most cases. Bacterial culture supernatants were unable to stimulate cytokine secretion. The higher bacterial concentrations (10^9 CFU ml^{-1}) led to a significant difference between the live and heat killed bacteria (p <0.05). L. bulgaricus LB-39 induced a significant increase of TNF-α, whereas seven *L. bulgaricus* strains produced no effect. The addition of *P. gingivalis* led to a significant increase in TNF-α in the culture

medium and the concentration increased during the first 2 hours of incubation, whereas at the end of the experiment the detected values were lower. When the epithelial cells were pretreated with lactobacilli prior to the *P. gingivalis* addition the concentration of secreted TNF-α was lower than when *P. gingivalis* was added alone to the cells maintained in the culture medium.

Hence in our present study we analyzed the secretion of two common proinflammatory cytokines that are generally associated with inflammation. Cytokines are secreted proteins that are responsible for many of the cellular responses of the innate and adaptive immunity, and thus function as the "messenger molecules" of the immune system. IL-8 and TNF-α are released by the oral epithelium in response to fungal or bacterial infection and they trigger further cellular responses. Cytokine expression induced by lactobacilli at various mucosal sites has been investigated in animals, human biopsy specimens, as well as in monolayer cell culture models. The inhibition of secretion of IL-4, IL-5, and IL-8 has been defined as a property of many strains of lactobacilli (Carbo et al., 2002; Pochard et al., 2002). However, the oral epithelium has not yet been investigated regarding cytokine expression after probiotic challenge. Because of the emerging concern of probiotic safety, especially in cases with immunocompromised patients, the results obtained by us merit particular interest. It is noteworthy to point out that probiotic properties do not always require administration of live bacteria. Additionally, fermented dairy products are common vehicles for probiotics and the oral cavity obviously serves as the first site where these bacteria can exert their effects. Subsequently our results provide further evidence showing that higher doses of live probiotic or putative probiotic species may induce IL-8 secretion similar to what was observed with the periodontal pathogen *A. actinomycetemcomitans*.

By including *P. gingivalis* in the study we investigated whether probiotic interaction with the epithelial cells affects further cell response caused by the *P. gingivalis*. This organism is an established periodontal pathogen that in addition to its large array of virulence factors inducing periodontal tissue damage may also possess a variety of evasion mechanisms towards host defense. Among others these may lead to altered polymorphonuclear cell (PMN) function and impaired immune response in general. We have demonstrated here that the epithelial cells pretreated with lactobacilli produced pronounced levels of IL-8, shortly after the addition of *P. gingivalis* suspensions and also displayed absence of IL-8 in the cell culture supernatant. This phenomenon could be attributed to the high proteolytic activity of *P. gingivalis* which causes degradation of cytokines and chemokines (Calkins et al., 1998; Banbula et al., 1999; Bodet et al., 2005). A strain dependent pattern on TNF-α secretion after *P. gingivalis* challenge was also observed. The strongest inducer of TNF-α among the *L. bulgaricus* strains, LB-39, led to significantly lower levels of TNF-α after incubation with *P. gingivalis*. Attenuated expression, but not absence of TNF-α after the *P. gingivalis* infection, was observed with another *L. bulgaricus* strain, namely LB-80. On the other hand, pretreatment of the epithelial cells with heat killed *L. bulgaricus* LB-42 led to six fold increase in TNF-α concentrations after *P. gingivalis* challenge. These results provide evidence to the complex mechanisms of interaction between lactobacilli and epithelial cells and warrant further investigations.

6 Key findings and conclusions

These series of studies addressed some key characteristics for the evaluation of probiotic properties of *L. delbrueckii* subsp. *bulgaricus* with respect to the oral cavity. The main findings can be summarised as follows:

1. Among *L. delbrueckii* subsp. *bulgaricus* species there are strains capable of inhibiting growth of some key oral pathogens, *S. mutans* and *A. actinomycetemcomitans* being the most susceptible to the inhibitory effect.

2. The adhesive properties of yogurt starter *L. delbrueckii* subsp. *bulgaricus* to saliva-coated surface are comparatively low although single strains demonstrated adhesive potential similar to that of strongly adhering reference species.

3. *L. delbrueckii* subsp. *bulgaricus* strains are harmless to main components of extracellular matrix, being unable to convert proMMP-9 to its active form, thus highlighting their safety on regulatory enzymes and structures of the host extracellular matrix.

4. *L. delbrueckii* subsp. *bulgaricus* can induce IL-8 and TNF-α after stimulation of oral epithelial cells *in vitro* which is strain and concentration dependent. The addition of *P. gingivalis* to epithelial cells pretreated with lactobacilli led to pronounced reduction of cytokine levels in cell culture supernatants probably due to its high proteolytic activity.

Based on the results of this research it is suggested that among the *L. delbrueckii* subsp. *bulgaricus* species there are strains that could be further studied as probiotics with eventual health promoting effects in the oral cavity. Furthermore, a combination of several strains with favorable properties merits further investigation in the oral health perspective. However, more research is needed to optimize the selection of proper strain/s to be used as oral probiotics and to decide the best and appropriate means for probiotic administration into the mouth. Phase I, II, III, and IV clinical trials need then to be conducted.

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Iva Stamatova

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