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PROBIOTIC LACTOBACILLI AND BIFIDOBACTERIA IN THE MOUTH

- *in vitro* Studies on Saliva-mediated Functions and Acid Production

by

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ABSTRACT

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Probiotic lactobacilli and bifidobacteria in the mouth – *in vitro* studies on saliva-mediated functions and acid production

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Probiotics are viable bacteria which, when used in adequate amounts, are beneficial to the health of the host. Although most often related to intestinal health, probiotic bacteria can be found also in the mouth after consumption of products that contain them. This study aimed at evaluating the oral effects of probiotic bacteria already in commercial use.

In a series of *in vitro* studies, the oral colonisation potential of different probiotic bacteria, their acid production and potential saliva-mediated effects on oral microbial ecology were investigated. The latter included effects on the salivary pellicle, the adhesion of other bacteria, and the activation of the peroxidase system. *Streptococcus mutans, Streptococcus gordonii*, *Aggregatibacter actinomycetemcomitans* and *Helicobacter pylori* were used as bacterial indicators of the studied phenomena.

There were significant differences between the probiotic strains in their colonisation potential. They all were acidogenic, although using different sugars and sugar alcohols. However, their acid production could be inhibited by the peroxidase system. Based on the results, it can be suggested that probiotic bacteria might influence the oral microbiota by different, partly species or strain-specific means. These include the inhibition of bacterial adhesion, modification of the enamel pellicle, antimicrobial activity, and activation of the peroxidase system.

To conclude, probiotic strains differed from each other in their colonisation potential and other oral effects as evaluated *in vitro*. Both positive and potentially harmful effects were observed, but the significance of the perceived results needs to be further evaluated *in vivo.*

Key words: probiotic, *Lactobacillus, Bifidobacterium,* saliva, oral microbiology, adhesion, enamel pellicle, peroxidase, streptococci, *Helicobacter pylori*

TIIVISTELMÄ

Anna Haukioja

Probioottiset laktobasillit ja bifidobakteerit suussa - *in vitro* tutkimus sylkivälitteisistä toiminnoista ja hapontuotosta

Hammaslääketieteen laitos, kariesoppi ja Suun terveystieteiden tutkijakoulu (PeGaSOS), Turun yliopisto, Suomi, 2009

Probiootit ovat eläviä bakteereja, jotka riittävässä määrin käytettynä ovat hyödyllisiä isännän terveydelle. Vaikka probiootit useimmiten liitetään suoliston terveyteen, niitä voi löytyä myös suusta probiootteja sisältävien tuotteiden käytön yhteydessä. Tämän tutkimuksen tavoitteena oli arvioida jo kaupallisessa käytössä olevien probioottien vaikutuksia suussa.

Sarjassa laboratoriotutkimuksia tutkittiin eri probioottikantojen kykyä kolonisoitua suuhun, niiden hapontuottoa sekä mahdollisia syljen välittämiä vaikutuksia suun mikrobiekologiaan. Näitä ovat vaikutukset sylkipellikkeliin, muiden bakteerien tarttumiseen sekä mahdollinen peroksidaasisysteemin aktivoiminen. Indikaattoribakteereina käytettiin *Streptococcus mutansia, Streptococcus gordoniita*, *Aggregatibacter actinomycetemcomitansia* ja *Helicobacter pyloriita*.

Kolonisaatiopotentiaalissa probioottikantojen välillä oli suuria eroja. Ne olivat kaikki happoja tuottavia, vaikka käyttivätkin eri sokereita ja sokerialkoholeja. Tulosten perusteella voidaan olettaa, että probiootit voivat vaikuttaa suun mikrobistoon monin eri tavoin ja että vaikutukset riippuvat osittain probioottibakteerilajista tai -kannasta. Havaittuja vaikutuksia olivat muiden bakteerien sitoutumisen estäminen, hampaan kiilteen päälle muodostuvan pellikkelin muokkaaminen, antimikrobinen aktiivisuus sekä peroksidaasisysteemin aktivoiminen.

Johtopäätöksenä voidaan todeta, että eri probioottikannat erosivat toisistaan sekä kolonisaatiopotentiaaliltaan että muilta mahdollisilta vaikutuksiltaan suussa, kun niitä tutkittiin laboratoriossa. Sekä hyodyllisiä että mahdollisesti haitallisia vaikutuksia havaittiin, mutta saatujen tulosten merkitysen arviointiin tarvitaan lisätutkimuksia elävässä eliössä.

Avainsanat: probiootti, *Lactobacillus*, *Bifidobacterium*, sylki, suun mikrobiologia, adheesio, kiillepellikkeli, peroksidaasi, streptokokit, *Helicobacter pylori*

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ABBREVIATIONS

BSA	Bovine serum albumin
CFU	Colony forming unit
DTT	Dithiothreitol
FMM	Fermentation minimal medium
GI	Gastro-intestinal tract
GCF	Gingival crevicular fluid
H_2O_2	Hydrogen peroxide
HA	Hydroxyapatite
$HOCl/OCl^-$	Hypochlorous acid/Hypochlorite
$HOSCN/OSCN^-$	Hypothiacyanous acid/Hypothiocyanite
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
Ig	Immunoglobulin
L.	*Lactobacillus*
Lc.	*Lactococcus*
LPO	Lactoperoxidase
LPO system	LPO, KSCN and H_2O_2
MPO	Myeloperoxidase
MRS	de Man, Rogosa and Sharpe medium
MTW	Microtitre well
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMN	Polymorphonuclear neutrophil
RT	Room temperature (20 - 23ºC)
SCN^-	Thiocyanate
SD	Standard deviation
SPO	Salivary peroxidase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-V.

I Haukioja A, Yli-Knuuttila H, Loimaranta V, Kari K, Ouwehand AC, Meurman JH and Tenovuo J: Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*. *Oral Microbiol Immunol* 2006: **21**: 326–332

II Haukioja A, Söderling E and Tenovuo J: Acid production from sugars and sugar alcohols by probiotic lactobacilli and bifidobacteria *in vitro. Caries Res* 2008: **42**:449-453

III Haukioja A, Loimaranta V and Tenovuo J: Probiotic bacteria affect the composition of salivary pellicle and streptococcal adhesion *in vitro. Oral Microbiol Immunol* 2008: **23:** 336 - 343

IV Haukioja A, Ihalin R, Loimaranta V, Lenander M and Tenovuo, J: Sensitivity of *Helicobacter pylori* to an innate defence mechanism, the lactoperoxidase-system, in buffer and in human whole saliva. *J Med Microbiol* 2004; **53:** 855 - 860

V Haukioja A, Loimaranta V and Tenovuo J: Activation of the lactoperoxidase – thiocyanate - hydrogen peroxide antimicrobial system by probiotic *Lactobacillus* strains (Manuscript)

1. INTRODUCTION

An increasing number of products containing probiotic bacteria are commercially available and used by consumers. Most of these products are consumed orally and hence the bacteria, or other microbes, in them are in contact with the oral surfaces. Indeed, some of the probiotic bacteria can be found in the oral cavity after consumption of products containing them (Yli-Knuuttila *et al.*, 2006, Caglar *et al.*, 2009).

Several studies have shown that probiotic bacteria may influence the oral microbial ecology (Hatakka *et al.*, 2007, Caglar *et al.*, 2005, Mayanagi *et al.*, 2009), but the effects of various probiotic bacteria in the oral cavity are difficult to predict. On the one hand, the efficient production of organic acids, which is a common characteristic of both lactobacilli and bifidobacteria, may be harmful in the mouth (Matsumoto *et al.*, 2005). On the other hand, however, probiotic *Lactobacillus* and *Bifidobacterium* strains may have effects potentially beneficial to oral health (Caglar *et al.*, 2005, Näse *et al.*, 2001, Shimauchi *et al.*, 2008). In addition to oral diseases, probiotic bacteria in the oral cavity may also have a broader influence on health by interacting with other microbes and saliva. After all, the human mouth has a complex microbiota, in which microbes that are usually associated with other parts of the GI tract are also present.

The current study was initiated to evaluate the oral effects of probiotic bacteria already in commercial use, with the emphasis being on the cariological perspectives.

2. REVIEW OF THE LITERATURE

2.1. The mouth as a microbial habitat

2.1.1. The oral cavity

The human mouth is an ideal environment for a variety of micro-organisms. Firstly, the temperature is rather constant, between 35 – 36ºC. Secondly, saliva bathes the oral surfaces, influencing the mouth as a microbial habitat in numerous ways: it keeps the surfaces moist and the pH relatively constant between 6.75 and 7.25, optimal for many microbes. Furthermore, saliva provides nutrients and salivary pellicle receptors for microbial attachment. Saliva also plays a key role in the oral defence against microbial overgrowth.

On the other hand, the human mouth contains distinct habitats, each of which supports the growth of a characteristic microbiota (Marsh & Martin, 1999, Mager *et al.*, 2003, Papaioannou *et al.*, 2009). The mucosal surfaces are regionally specialised, thus for example the palate and the papillary structure of the dorsum of the tongue provide different niches for microbes. Teeth, unique for the mouth as non-shedding surfaces, provide several distinct surfaces, too. In addition, the front teeth are exposed to air containing approximately 21% of oxygen, whereas the oxygen tension of the buccal folds of the upper and lower jaw is only 0.3–0.4% (Marsh & Martin, 1999). Accordingly, the redox potentials of different sites in the oral cavity differ significantly. Furthermore, the gingival crevicular fluid (GCF) and saliva provide different nutrients and essential co-factors for microbes to grow. The resident microbiota has a significant influence on the micro-environment by modifying the environment, making it suitable for the more fastidious species.

Diverse defence mechanisms against microbes operate in the oral cavity (for a review, see Cole & Lydyard, 2006). The integrity of the enamel, covered by the acquired enamel pellicle, and the oral mucosa, covered by mucus, act as the first line of defence, as physical barriers which prevent the penetration of microbes. Desquamation of the epithelium is important in limiting the colonisation of bacteria on the epithelial cells. The innate host defence in the oral cavity consist of cellular mechanisms, including the natural killer cells and the phagocytotic cells, PMNs and macrophages, numerous soluble defence factors of the saliva (see 2.1.2.3.) and GCF, and defence proteins and peptides produced by the epithelial cells. The epithelial cells express Toll-like receptors and thus produce various cytokines and β-defensins upon activation. Both the humoral and cell-associated responses of the adaptive immunity function in the oral cavity: both CD4 and CD8-positive T cells can be found in the epithelium and saliva. Saliva and GCF contain Igs derived from serum or produced locally near the mucosal surfaces and the acini of salivary glands. The defence factors in the saliva are discussed in greater detail in Section 2.1.2.3.

Microbes are removed from the oral cavity continuously by processes such as salivary clearance, mastication and oral hygiene. Despite this, numerous bacterial species can be found at any oral site and more than 700 different species or phylotypes of bacteria have been detected in the oral cavity (Aas *et al*., 2005).

2.1.2. Saliva

2.1.2.1. Oral fluid or whole saliva

Saliva is the common word used instead of such expressions as oral fluid or whole or mixed saliva to describe the combined fluids in the mouth. Whole saliva is a mixture of pure glandular salivas, gingival crevicular fluid, oral epithelial cells, micro-organisms and their by-products, food, etc. The functions of saliva can be related either to its fluidity, including lubrication or clearance, or to specific component(s), such as the defence proteins or buffering capacity. The composition of saliva is not stable but changes, for example, during ageing, in various physiological situations such as pregnancy and due to different diseases or medications (Laine *et al.*, 1988, Johnson *et al.*, 2000, Aps & Martens, 2005). Furthermore, saliva varies in different parts of the oral cavity (Sas & Dawes, 1997). Individual differences in saliva composition are large, and the composition of saliva is dependent on the salivary flow rate with some salivary components exhibiting a circadian rhythm (Ferguson & Botchway, 1979).

2.1.2.2. The salivary pellicle

The salivary pellicle is an aqueous protein film covering all oral surfaces. As soon as a tooth erupts, or minutes after removal of this film, the salivary pellicle is reformed on tooth surfaces (Skjorland *et al.*, 1995, Vacca Smith & Bowen, 2000). On enamel surfaces, the pellicle is formed by selective adsorption of proteins, peptides and possibly also other constituents such as phospholipids present in the whole saliva (Slomiany *et al.*, 1986, Al-Hashimi & Levine, 1989, Siqueira *et al.*, 2007, Siqueira & Oppenheim, 2009). It has been assumed that the primary protein layer is formed by proteins binding to calcium and phosphate ions, the components of hydroxyapatite, and a third group of proteins in the pellicle consists of proteins able to interact with other proteins (Siqueira *et al.*, 2007). On the other hand, adsorption of salivary globes, globular protein aggregates or micelle-like structures, may bring all types of proteins, not only those adhering well on hydroxyapatite, into the pellicle in the early phase of pellicle formation (Vitkov *et al.*, 2004). The pellicle is formed in less than one minute, and it has been estimated that the full thickness of the pellicle is reached within an hour *in vivo* (Skjorland *et al.*, 1995). Still, it continues to mature for hours or even days (Hannig, 1999). Furthermore, continuous adsorption and desorption of proteins as well as conformational or proteolytic changes in the attached proteins make the pellicle a dynamic and ever-changing structure (Bennick *et al.*, 1983, Hannig, 1999, Siqueira & Oppenheim, 2009).

The composition of the pellicle is affected by both the substrate and the surface. Thus, the protein composition on different parts of dentition (Carlen *et al.*, 1998) and on the enamel surface or on different prosthetic materials is different (Lee *et al.*, 2001, Svendsen & Lindh, 2009). In addition to proteins from salivary glands, serum proteins and bacterial components attach to the enamel pellicle (Vacca Smith & Bowen, 2000, Carlen *et al.*, 2003, Siqueira *et al.*, 2007). Furthermore, diet and the use of oral hygiene products may influence the composition of the pellicle (Vacca Smith & Bowen, 2000). There are also individual differences in the pellicle composition (Bruvo *et al.*, 2009). Only 14% of the known proteins in enamel pellicles formed *in vivo* are estimated to be of salivary gland origin, although this may be an underestimation (Siqueira *et al.*, 2007).

Although non-salivary, i.e. serum, cell and bacterial proteins, significantly outnumber the salivary gland constituents in the enamel pellicle, it has been supposed that the bulk of the pellicle structure is formed of salivary proteins (Siqueira *et al.*, 2007).

The most heavily studied functions of the enamel pellicle are the mediation of adherence of oral bacteria and the protection of the enamel against the de-mineralisation process. Proteins with antimicrobial properties which are involved in buffer capacity, remineralisation or lubrication have been identified (Pruitt & Adamson, 1977, Leinonen *et al.*, 1999, Li *et al.*, 2004b). The protection of the enamel seems to occur due to the slower dissolution rate of the hydroxypapatite when covered by the pellicle, but there are individual differences in the efficacy of the protection (Hannig *et al.*, 2004, Bruvo *et al.*, 2009). Pellicle proteins with calcium binding domains may be important in the re-mineralisation process (Siqueira *et al.*, 2007). The role of the different protective enzymes in the salivary pellicle is difficult to estimate: Several enzymes bind to the pellicle in their active form, suggesting therefore a role in defence against microbes or in buffering (Li *et al.*, 2004b). Yet, some of the defence proteins can be less active in the pellicle due to changes in their conformation or inhibition by substances found in the whole saliva (Hannig *et al.*, 2004, Hannig *et al.*, 2008). Furthermore, lactoferrin, which is an antimicrobial agent in the liquid phase of saliva, may support the binding of some streptococcal strains to the salivary pellicle (Rudney *et al.*, 1999).

2.1.2.3. Antimicrobial properties of saliva

Saliva protects the oral cavity, teeth and mucosal surfaces, in various ways: Perhaps the most important single property of the saliva is the continuous flushing of oral surfaces. Saliva dilutes harmful compounds, and microbes and other damaging particles not attached to oral surfaces are rapidly swallowed.

Saliva contains numerous innate defence proteins and peptides with various antimicrobial activities including agglutination, bactericidal, bacteristatic and anti-adhesive activities (Cole & Lydyard, 2006). These proteins are not specific but work against a wide variety of bacteria, viruses and fungi. It seems likely that the main function of these defence proteins is to prevent the unrestricted growth of microbes in the oral cavity. The amount of most of the innate components reaches the adult level already in early childhood, although some changes in the levels appear to occur in puberty (Tenovuo *et al.*, 1986, Kirstilä *et al.*, 1998). The salivary peroxidase system, important to this thesis, is discussed in greater detail in the next section.

The acquired immunity in saliva consists of secretory IgA, synthesised locally by plasma cells in the vicinity of the glandular acini and oral mucosa, and of IgG, IgM and IgA via GCF, into which they are filtrated from the serum or produced by the plasma cells in the gingiva. It has also been proposed that IgG and monomeric IgA are transmitted through the oral mucosa, possibly in amounts depending on the integrity of the epithelial barrier (Välimaa *et al.*, 2002, Brandtzaeg, 2007). Secretory IgA is the main immunoglobulin in saliva; its main function is thought to be in enhancing the clearance of bacteria by acting as an effective agglutinin and by preventing bacterial adherence. In addition, it acts by neutralising viruses, toxins and bacterial enzymes. As it does not activate complement, it is considered to be an anti-inflammatory immunoglobulin (Cole & Lydyard, 2006). Only

little is known about the role of other immunoglobulins in oral health. Salivary IgG has been suggested to be responsible for the HSV-neutralising activity of saliva (Välimaa *et al.*, 2002).

The significance of a single specific defence protein is difficult to predict. Salivary proteins may be amphifunctional, that is, distinct functions of same protein benefit the bacteria and the host. Redundancy refers to fact that many of the salivary proteins exhibit similar functions *in vitro*. It seems that *in vivo*, the different defence factors compensate for each other if one of the components is missing. For example, individuals with an IgA deficiency do not have more oral diseases than healthy individuals but do have, instead, significantly higher levels of IgM and normal or possibly even higher levels of innate defence proteins in their saliva (Kirstilä *et al.*, 1994, Nikfarjam *et al.*, 2004). Furthermore, different defence proteins seem to work in an additive or even synergistic manner. Thus, the effects of the peroxidase system are enhanced by IgA, lysozyme and lactoferrin, more than the combined effects of these factors alone would suggest (Tenovuo *et al.*, 1982, Lenander-Lumikari *et al.*, 1992, Soukka *et al.*, 1991). Finally, many of the defence proteins are multifunctional, e.g. lactoperoxidase inhibits both the glucose and oxygen metabolism and the adhesion of *Streptococcus mutans* (Roger *et al.*, 1994, Månsson-Rahemtulla *et al.*, 1987, Carlsson *et al.*, 1983).

2.1.3. Peroxidase activity in the human saliva

2.1.3.1. Peroxidase enzymes

Peroxidase enzymes in human saliva, myeloperoxidase (MPO) and salivary peroxidase (SPO), belong to the group of mammalian hemeperoxidases. This group of peroxidase enzymes consists of four superfamilies of different enzymes, which catalyse a reaction between a halide or pseudohalide and a hydrogen peroxidase. The reaction generates an oxidised halide or pseudohalide. Myeloperoxidase can use Cl^-, Br^-, I^- and SCN^- ions as a substrate, whereas salivary peroxidase can oxidise only Br^-, I^- and SCN^- ions. The oxidation products of the enzyme reactions are HOCl, HOBr, HOI and HOSCN, respectively, in equilibrium with their unprotonated forms. Although there is still some doubt about the oxidation products of SCN^-, they are termed $HOSCN/OSCN^-$. (Davies *et al.*, 2008)

SPO is produced in the parotid and submandibular salivary glands (Riva *et al.*, 1978) but MPO enters the saliva via the gingival crevicular fluid, into which it is released from polymorphonuclear (PMN) leukocytes. The concentration of SPO is rather constant, around 2 µg/ml (Thomas *et al.*, 1994), whereas the amount MPO is dependent on gingival health. During an inflammation, the PMN leukocytes are recruited to the gingival sulcus, where they are subjected to osmotic lysis and thus excrete their contents into the sulcus. The total amount of peroxidases in the whole stimulated saliva of a healthy adult is around 5 µg/ml (Thomas *et al.*, 1994). On average, MPO is responsible for 75% of the total peroxidase activity in the mixed saliva. The two enzymes are distributed unevenly: SPO is found in the soluble portion and almost 80% of MPO is found in the salivary sediment (Thomas *et al.*, 1994).

In saliva, both SPO and MPO oxidise SCN^- and generate $HOSCN/OSCN^-$ (van Dalen *et al.*, 1997, Thomas & Fishman, 1986). SCN^- is the preferred substrate because the

concentration of SCN^- in saliva is high, it varies between 0.5 and 6 mM, the median being around 1 mM (Tenovuo & Mäkinen, 1976), and the reaction rate is significantly faster than with the other substrates (Furtmuller *et al.*, 1998, Furtmuller *et al.*, 2002). The concentrations of the other potential substrates are significantly lower than that of SCN^- (Anttonen & Tenovuo, 1981). $HOSCN/OSCN^-$ is also generated in the reaction between SCN^- and $HOCl/OCl^-$ formed in the gingival sulcus (Fig. 1). The role of Cl^- is significant in the gingival crevice as its concentration is over 2000 times higher than that of SCN^- (Fig. 1) (Anttonen & Tenovuo, 1981). Furthermore, I^- may have a greater role as a substrate for peroxidases in saliva than previously suggested if it is already oxidised in the ducts of the salivary glands (Geiszt *et al.*, 2003). $HOSCN/OSCN^-$ is also released into the saliva directly from the parotid ducts (Pruitt *et al.*, 1983, Geiszt *et al.*, 2003). Hydrogen peroxide needed for the oxidation reaction in the saliva is generated by some oral microbes, PMN leukocytes and also, in ducts of the parotid glands, by Duox enzymes. The latter are NADPH oxidase homologues present on several mucosal surfaces (Geiszt *et al.*, 2003).

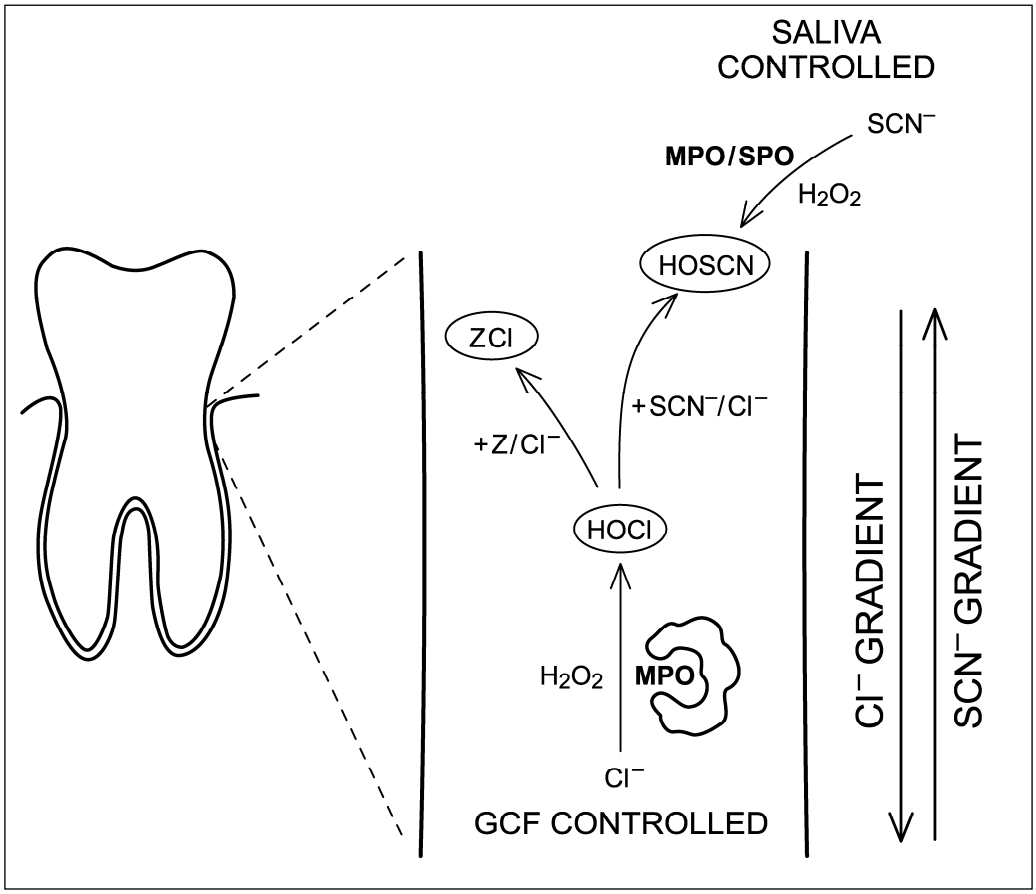

Figure 1. Formation of HOSCN and HOCl in saliva and in gingival crevicular fluid. HOCl can also react with small molecules other than SCN^- to produce secondary antimicrobials (ZCl). Modified from Ashby (2008).

Human SPO and lactoperoxidase (LPO) are encoded by the same gene, but transcript variants encoding different isoforms are found (Månsson-Rahemtulla *et al.*, 1988, Ueda *et al.*, 1997, Kiser *et al.*, 1996, Shin *et al.*, 2000, Fragoso *et al.*, 2009). The different isoforms may be related to the expression of the enzymes in different tissues or physiological conditions (Fragoso *et al.*, 2009). The enzyme activities of the different isoforms, however, are similar (Fragoso *et al.*, 2009). The salivary peroxidase and lactoperoxidases also seem to be very similar in all mammalian species. Bovine LPO and human SPO share an 83% amino acid identity, although SPO contains more proline and serine residues than LPO (Månsson-Rahemtulla *et al.*, 1988). They also differ in their glycosylation, both in carbohydrate composition and content. Catalytically, bovine LPO and human SPO are very similar, at least in respect of peroxidation of SCN^- (Pruitt *et al.*, 1988). The pH-independent rate constants describing the formation of the enzyme-substrate complexes are similar, only the pH-dependent equilibrium constant for substrate inhibition is twice as high for SPO than for LPO (Pruitt *et al.*, 1988, Månsson-Rahemtulla *et al.*, 1988). The efficacy of both enzymes is dependent on pH, and both are inhibited by high SCN^- concentrations at lower pH values (Pruitt *et al.*, 1988). However, the optimum pH for both enzymes is acidic in physiological SCN- concentrations (Pruitt *et al.*, 1988). Thus, despite the small differences in structure and catalytic activity, bovine LPO serves well as a model in studies of peroxidase-mediated functions of human saliva.

2.1.3.2. Its biological role

Peroxidase enzymes in the saliva have a dual biological role. First, their oxidation products are antimicrobial, and second, they protect the host tissues from the toxicity of H_2O_2.

The antimicrobial activity of the peroxidase system was first described in bovine milk and later in human secretions including saliva, cervical mucus and tears (Tenovuo, 1985). Today, the peroxidase systems have been recognised as an important defence factor in many, if not all, mucosal secretions. Physiological concentrations of $HOSCN/OSCN^-$ provide antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria, viruses and fungi (Lumikari *et al.*, 1991, Lenander-Lumikari, 1992, Mikola *et al.*, 1995, Ihalin *et al.*, 2001). In general, Gram-negative bacteria are more sensitive to $HOSCN/OSCN^-$ than Gram-positive bacteria (Marshall & Reiter, 1980, Ihalin *et al.*, 2001). Variability in sensitivity has been observed between related species, or even strains of the same species (Carlsson *et al.*, 1983, Ihalin *et al.*, 2003). These differences may be related to the presence or activity of an oxidoreductase, which converts $OSCN^-$ to SCN^- (Carlsson *et al.*, 1983). Furthermore, a unique stress response towards $HOSCN/OSCN^-$ has been reported in *Escherichia coli* (Sermon *et al.*, 2005). The peroxidase system inhibits crucial bacterial metabolic activities, such as glucose and oxygen metabolism of streptococci (Månsson-Rahemtulla *et al.*, 1987, Carlsson *et al.*, 1983) and urease activity of *H. pylori* (Shin *et al.*, 2002).

Sulfhydryl groups in proteins or other molecules are the main target of $HOSCN/OSCN^-$ (Thomas & Aune, 1978a), but it has been suggested that aromatic residues and amine groups in amino sugars, phospholipid head groups and nucleobases may be targeted, too (reviewed by Davies *et al.*, 2008). Thus, the internalisation is important for the

antimicrobial activity of HOSCN/$OSCN^-$. HOSCN and $OSCN^-$ are in an equilibrium, and the equilibrium reaction has a pK_a of 5.3 (Thomas, 1981). Hence, bacteria are more sensitive to the HOSCN/$OSCN^-$ in an acidic environment, since the protonated and accordingly uncharged HOSCN diffuses readily into the cells, where it can react with intracellular proteins. Furthermore, due to their more permeable cell wall, Gram-negative bacteria are more sensitive to the HOSCN/$OSCN^-$ than are Gram-positive bacteria. Alterations in cell envelope, such as removal of lipopolysaccharide from the outer membrane or mutations which make the bacterial cell wall more permeable, also increase the susceptibility of the bacteria to HOSCN/$OSCN^-$ (Thomas & Aune, 1978b, Purdy *et al.*, 1983).

LPO and human SPO retain their enzymatic activities when bound to hydroxyapatite, the enamel pellicle or to bacterial surfaces *in vitro* (Pruitt & Adamson, 1977, Tenovuo *et al.*, 1977, Pruitt *et al.*, 1979, Hannig *et al.*, 2008). This may influence the role of the peroxidases in oral biofilms: the pellicle-bound peroxidase could influence the initial formation of the dental plaque. In addition, a peroxidase bound to a bacterial surface brings HOSCN/$OSCN^-$ close to the microbial target. Thus, the concentration of HOSCN/$OSCN^-$ can be much higher in oral biofilms and in the vicinity of the microbial target compared to the whole saliva (Cole *et al.*, 1981). However, recent evidence suggests that although human SPO in its active form binds into an in situ-formed pellicle, its activity may decrease rapidly *in vivo* (Hannig *et al.*, 2008). On the other hand, *in vitro* evidence suggests that the LPO protein itself, without substrates, may prevent the adhesion of *S. mutans* and inhibit the streptococcal glucosyltransferases (Roger *et al.*, 1994, Korpela *et al.*, 2002). In addition, individuals whose saliva does not support streptococcal adherence have elevated levels of peroxidase in their saliva (Rudney *et al.*, 1999).

In addition to their antimicrobial activity, peroxidase enzymes protect the host tissues from the toxicity of H_2O_2 and possibly contribute to the protection of the host by detoxifying or inactivating some carcinogenic or mutagenic compounds (Tenovuo, 1986). Furthermore, new functions of LPO and HOSCN/$OSCN^-$ have been described recently. It has been suggested that HOSCN could induce NF-κB-regulated signalling, and orally administered LPO could be involved in the regulation of gene expression in the small intestine (Wang *et al.*, 2006, Wakabayashi *et al.*, 2007).

2.2. Oral microbiology

2.2.1. Development of the oral microbiota in children

As soon as a baby is born, the bacterial colonisation of the mouth begins. In addition to frequent contact and sufficient inoculation size, the colonisation by oral bacteria requires a suitable attachment site and nutrients for growth. For example, children whose mouths are colonised by *S. mutans* are more likely to have been exposed to behaviour which allows for contact with adults' saliva and frequent sugar exposure than children without *S. mutans* (Wan *et al.*, 2001).

The most important source of oral microbes is the saliva, especially from mothers, fathers or siblings. Information about the early development of oral microbiota is scarce,

but according to culture-based methods, acquisition of microbes from the birth canal is of limited significance, only *S. epidermis* acquired at birth seems to persist for a longer time (Kostecka, 1924, Hegde & Munshi, 1998). On the other hand, viruses seem to be able to infect the child during delivery (Puranen *et al.*, 1997, Gaillard *et al.*, 2000). The very first bacterial colonisers of the oral cavity are viridans streptococci; they are present already 8 hours after birth (Rotimi & Duerden, 1981). During the first days, the number of species increases rapidly, (Rotimi & Duerden, 1981, Makhoul *et al.*, 2002). Tooth eruption around the age of six months changes the oral cavity significantly. Teeth are a non-shedding surface, and the sulcus area brings another new ecological niche into the oral cavity. In addition, the diet changes simultaneously with tooth eruption. New bacterial genera begin to colonise the mouth, and by the age of 3 years children have a multiform oral microflora, including also Gram-negative anaerobic species (Könönen *et al.*, 1994, Papaioannou *et al.*, 2009). Lactobacilli and bifidobacteria, which are resident species in the oral cavity, are discussed in Sections 2.4.1. and 2.4.2.

Earlier culture-based studies suggested that the teeth and the sulcus area are essential for the colonisation of some microbes associated with caries and periodontal diseases. Accordingly, some bacterial species, such as *S. mutans* and *S. sanguinis*, were suggested to colonise at a discrete time period, called the window of infectivity (Caufield *et al.*, 1993, Caufield *et al.*, 2000). However, more recent studies indicate that the *S. mutans* colonisation may already occur prior to tooth eruption (Wan *et al.*, 2001), and the acquisition of this bacterium is also possible later (Straetemans *et al.*, 1998). Several anaerobic species are a part of the oral microbiota in healthy children, although they seem to be a part of the transient microbiota, at least during first years of life. However, even if the species are persistent, the turnover is high at strain level (Könönen *et al.*, 1994, Lamell *et al.*, 2000, Haraldsson *et al.*, 2004).

After tooth eruption, changes in microbiota are much more infrequent until the next major physiological changes occur in puberty. Although the salivary flow rate is not significantly affected, changes occur in the salivary composition. The mouth will become more susceptible to caries as the buffer capacity of the saliva decreases (Söderling *et al.*, 1993, Bruno-Ambrosius *et al.*, 2004). Hormonal changes may also affect sub-gingival microbiota, leading to a higher tendency towards gingivitis (Gusberti *et al.*, 1990, Moore *et al.*, 1993). However, the menstrual cycle does not seem to have an effect on microbiota (Fischer *et al.*, 2008).

2.2.2. Adult oral microbiota

Although it has been estimated that over 500 different bacterial species can colonise the oral cavity, the number of species present in the oral cavity of a single individual is significantly lower. The number of predominant species in the oral cavity of a healthy adult seems to vary between 30 and 80 species (Aas *et al.*, 2005). Several bacterial species can be detected in most oral sites, while others are relatively site-specific (Mager *et al.*, 2003, Aas *et al.*, 2005). Colonisation by a new bacterial species is infrequent; in contrast, the adult oral microbiota is relatively stable even at strain level (Mättö *et al.*, 1996). However, new species are acquired as transmission of oral bacteria between spouses occurs (van Steenbergen *et al.*, 1993, Mättö *et al.*, 1996). In addition, changes

in the oral cavity may alter the bacterial microbiota. For example, the loss of teeth can lead to a loss of suitable habitat, as seen in the case of *S. mutans* being ubiquitous in dentate mouths but which cannot be cultured and is detected at a much lower level in the oral cavity of the elderly without dentures (Carlsson *et al.*, 1969). Similarly, changes in the salivary flow or in diet alter the mouth as a microbial habitat (Beighton *et al.*, 1999, Almstahl *et al.*, 2001).

Similarly to the other parts of the body, the stability of resident microbiota also protects the oral cavity from invading exogenous, potentially harmful microbes (Marsh, 2003). The phenomenon is called colonisation resistance. Colonisation resistance involves several mechanisms, such as occupation of adhesion sites, alteration of the physio-chemical environment, production of antagonistic substances and utilisation of available nutrients (Wilson, 2005). Each member within the microbial community has a functional role and, thus, the degree of colonisation resistance is likely to be a consequence of the interactions between all the microbes in the niche in question (Wilson, 2005). The stability of a mature oral microbiota can be demonstrated by the observation that it is more difficult to introduce new species into the oral cavity of older animals than to that of younger ones (Socransky & Manganiello, 1971). In addition, loss of colonisation resistance can lead to severe health problems in the oral cavity (Jobbins *et al.*, 1992).

2.2.3. Dental plaque, a biofilm on the tooth surface

Adhesion is a key step for microbial colonisation. Without adhering, or otherwise escaping the washing by the saliva, bacteria are rapidly swallowed. The biofilm formation on the teeth begins by the adhesion of certain microbial species, mainly streptococci and *Actinomyces* species, to the acquired pellicle (Li *et al.*, 2004a). Non-specific forces bring bacteria into close contact with the surface, allowing stronger, specific interactions between the bacterial adhesin and the receptor protein, as demonstrated with *S. mutans* strains with and without antigen I/II (Xu *et al.*, 2007). *In vitro* results indicate that the bacterial species binding to saliva-coated and to plain hydroxyapatite are different (Clark *et al.*, 1978), indicating that the pellicle at least initially determines the particular bacteria attaching to tooth surfaces. Thus, individual differences in receptor proteins which modulate the initial adhesion of bacteria to the oral surfaces may influence the individual's susceptibility to oral diseases, such as caries (Jonasson *et al.*, 2007). In addition to the salivary pellicle, intragenic coaggregation, unique to the primary colonisers, may be important in the initial adhesion (Kolenbrander *et al.*, 1990). The first colonisers play an important role in dental plaque formation by providing receptors for the attachment of other bacteria and also by modifying the environment.

After the primary colonisers are attached, further accumulation of plaque continues through the attachment of new species and the growth of the attached bacteria. Later colonisers adhere either to the already attached bacteria or to salivary molecules attached to them (Lamont & Rosan, 1990). In addition, extracellular glucans produced by the plaque bacteria facilitate interbacterial binding and accumulation (Rölla, 1989). A significant part of the plaque mass consists of exopolysaccharides produced by mutans and some mitis group streptococci (Minah & Loesche, 1977). Interestingly, expression of genes involved in the exopolysaccharide production by *S. mutans* is upregulated in

cells growing in a biofilm (Shemesh *et al.*, 2007). On the other hand, environmental factors, such as the availability of nutrients or activity of the host enzymes, influence the expression of biofilm-associated genes, and thus drive the shift towards the biofilm phenotype (Loo *et al.*, 2000, Klein *et al.*, 2009). Interactions among bacteria are also proposed to promote the maturation of the biofilm (Burmolle *et al.*, 2006, Filoche *et al.*, 2004, McNab *et al.*, 2003). Today, more than 1000 strains of human oral bacteria have been tested for their ability to co-aggregate (reviewed by Kolenbrander *et al.*, 2006). Extreme examples of co-aggregation ability are *Fusobacterium nucleatum* and *Prevotella loescheii* which act as bridges between the different bacterial species (Kolenbrander *et al.*, 1985, Kolenbrander *et al.*, 1989). As the plaque grows, the diversity of the species increases, although the streptococci may play a significant role in the total mass increase, especially in the supra-gingival plaque development (Haffajee *et al.*, 2008a, Dige *et al.*, 2009). Furthermore, the local environment and oral hygiene influence the selection of bacteria, so that not only are sub- and supragingival dental plaques different, but the composition of the dental plaque is also different in different parts of the dentition (Haffajee *et al.*, 2008b).

Dental plaque is a community of bacteria which exhibit synergistic and antagonistic interactions: for example, *in vitro* evidence suggests that the presence of anaerobic species is dependent on facultative species, which consume oxygen from the microenvironment (Bradshaw *et al.*, 1997), and nutritional interactions which facilitate the growth of the more fastidious species (Mikx & van der Hoeven, 1975, Bradshaw *et al.*, 1994). Again, coaggregation seems to benefit the interacting bacteria (Bradshaw *et al.*, 1998). Furthermore, the phenotypic heterogeneity of bacteria growing in biofilms also facilitates the survival of a single species bacterial community in temporally variable conditions (Goryachev *et al.*, 2005, Maamar *et al.*, 2007). The bacteria communicate through chemical signals (Egland *et al.*, 2004). If the signals are related to the cell density, the communication phenomenon is called quorum sensing. Based on *in vitro* studies, it can be suggested that in the oral cavity quorum sensing signals may play an integral part in biofilm formation (Yoshida *et al.*, 2005, Xie *et al.*, 2007). On the other hand, bacteria in biofilms may hinder each other by, for example, competing for nutrients or secreting antimicrobial substances (Hillman *et al.*, 1985, Hojo *et al.*, 2007b).

Bacteria in biofilms are often more resistant to shearing forces, host defence factors and antimicrobial compounds than bacteria growing in a planktonic phase. Antimicrobial agents are effective against forming biofilms, but the bacteria in the mature biofilm are more resistant than planktonic cells (Singh *et al.*, 2002, Yang *et al.*, 2006). The protection seems to be partly due to the biofilm structure: Tightly attached bacteria are difficult to remove, and the bacteria are protected by the exopolysaccharide matrix. The biofilm matrix can also contain extracellular enzymes which benefit the whole community of bacteria (Dibdin *et al.*, 1996). In addition, the concentrations of antimicrobial agents are dependent on diffusion of the compound into the biofilm (Watson *et al.*, 2005). The resistance of the biofilm bacteria can be related to nutrient limitation or a slow growth rate (Anderl *et al.*, 2003, Spoering & Lewis, 2001). The sensitivity of microbes to antimicrobial agents is often affected by the growth rate, the slower-growing or stationary-phase bacteria being less sensitive. This is true not only concerning antibiotics affecting the dividing bacteria but also regarding host defence factors such as the peroxidase system

(Purdy *et al.*, 1983). The proportion of 'persister cells', dormant, non-dividing cells with a slow metabolic activity, is increased in biofilms (Lewis, 2007). Although the antibiotic resistance of biofilm cells can be related to the biofilm phenotype, genetic transfer of the antibiotic resistance in biofilms may also occur *in vivo* (Warburton *et al.*, 2007).

2.2.4. Oral diseases with a microbiological aetiology

2.2.4.1. The ecological plaque hypothesis and oral diseases with a microbiological aetiology

Today, the ecological plaque hypothesis is the paradigm with which plaque-associated diseases are viewed. As discussed above, the mouth is sterile only in utero and the development of resident microbiota begins within hours of birth. Furthermore, bacterial species associated with oral diseases can also be found in a healthy mouth. Thus, the microbial aetiology of dental plaque-associated oral diseases is often difficult to define. According to the ecological plaque hypothesis, the selection of pathogenic species is due to the ecological pressure of the local environment. In addition, any species with relevant traits can contribute to the disease process. Thus, to prevent plaque-associated diseases, not only the potential pathogens but also the environmental stresses responsible for the selection of the pathogenic species may be targeted. (Marsh, 2003)

Oral diseases of a microbiological aetiology include the plaque-associated diseases, i.e. dental caries, gingivitis and periodontitis, and endodontic infections, fungal and viral infections, as well as different acute bacterial infections. Of these, the plaque-associated diseases and fungal infections have been in the focus of probiotic research. In addition to these diseases, oral malodour, which cannot be classified as a disease, has been a target of probiotic therapy. These conditions, thus relevant to this thesis, are briefly described below.

2.2.4.2. Dental caries

Dental caries can be defined as localised destruction of the tissues of the tooth by acids generated in the bacterial fermentation of dietary carbohydrates (Marsh & Martin, 1999). However, it is a multifunctional disease and in addition to the bacteria, the diet and host factors, for example, also strongly influence the disease outcome.

Consumption of fermentable carbohydrates results in acidification of the dental plaque (Stephan, 1944). This is mainly due to lactic acid generated by acidogenic bacteria in the dental plaque, although also other acids are among the metabolic end products (Margolis & Moreno, 1994). The pH of the dental plaque decreases rapidly after the exposure to sugar. In 15 to 20 minutes, the proportions of acids begin to return to the situation for resting or starved dental plaque, where acetic and propionic acids are the main organic acids of the plaque fluid (Margolis & Moreno, 1994). Acidic conditions favour acid tolerant, aciduric species (Horiuchi *et al.*, 2009). In addition, lactic acid may also increase the sensitivity of less aciduric oral bacteria to acidic conditions (Dashper & Reynolds, 2000), and the acidification of the dental plaque may lead to an adaptation to acids (Takahashi & Yamada, 1999). Repeated conditions of low pH in the plaque following a frequent intake of fermentable carbohydrates or inefficient clearance thus

results in an ecological shift suggested by the ecological plaque hypothesis (Marsh, 2003). Indeed, there is evidence that the acidic periods after sugar exposure in the dental plaque fluid of caries-positive people have lower pH and last longer than in caries-free people (Margolis & Moreno, 1992). The high lactic acid production and low minimum pH of the dental plaque after sugar exposure correlates with caries history, especially in children (Shimizu *et al.*, 2008).

The role of specific plaque bacteria in dental caries is again a topic of intensive research, as molecular methods allow simultaneous investigation of several bacterial species and not yet cultivable bacteria. However, already Miller (1889) suggested that caries is caused by lactic acid which is generated during the fermentation of starch and sugar contained in food remains in the mouth. He further proposed that "*Bacillus acidi lactici*" are the probable cause of dental caries. At the beginning of the 20th century, lactobacilli, particularly *L. acidophilus* [*Bacillus acidophilus*], were considered to be the causative agent of caries (see Bunting, 1933). Later, the role of lactobacilli was shown to be more associative than causative in the initiation of the dental caries, and together with *Actinomyces* and *Candida albicans*, they are associated more with the progression of the disease and with root caries (Boyar & Bowden, 1985, Bjorndal & Larsen, 2000, Brailsford *et al.*, 2001).

The role of *S. mutans* or the group of mutans streptococci in dental caries has been studied intensively since the 1960s. *S. mutans* is frequently isolated from caries lesions and it exhibits an ability to cause caries in experimental animals. It possesses a range of putative virulence factors – acidogenicity, aciduricity, the ability to adhere on tooth surfaces, and the production of exopolysaccharides. This has lead to *S. mutans* being designated the major pathogen in human dental caries. This was established in a systematic literature review (Tanzer *et al.*, 2001). However, it has been suggested that caries lesions can also develop in the absence of *S. mutans* (Hardie *et al.*, 1977). In fact, 10 – 20% of subjects with severe caries do not have detectable levels of *S. mutans* (Aas *et al.*, 2008). *S. sobrinus*, another species of the group of mutans streptococci, is detected infrequently in the absence of *S. mutans* (Babaahmady *et al.*, 1998). These findings support the ecological approach in comprehending dental caries (Kleinberg, 2002, Takahashi & Nyvad, 2008), and now, 120 years after Miller (1889), we are still discussing the microbiological aetiology, and the role of specific microbes, in tooth decay.

2.2.4.3. Periodontal diseases

Gingivitis and periodontitis are the most common diseases with a microbial aetiology affecting the periodontium. Gingivitis is an inflammation of the marginal periodontal tissues associated with an accumulation of dental plaque. The inflammation is reversible, and there is no destruction of the periodontal attachment of the teeth, although inflammation may be found at sites with a prior attachment loss. In contrast to gingivitis, periodontitis is characterised by a progressive destruction of the supporting structures of the teeth. It is the result of inflammatory responses to dental plaque in a susceptible host. Bacteria may also directly cause tissue damage due to virulence factors, such as toxins and enzymes (for a review see Smalley, 1994). Furthermore, the capacity of micro-organisms to

induce the production and / or activation of matrix metalloproteinases in host tissues is important in the pathogenesis of periodontitis (Okamoto *et al.*, 1997, DeCarlo *et al.*, 1997). The inflammatory response including an increased flow of GCF and a rise in pH favours the Gram-negative, proteolytic species thus leading to an ecological shift as suggested by the ecological plaque hypothesis (Marsh, 2003).

The microbiological aetiology of periodontitis is not simple. Both the persistence of pathogenic species and the absence of beneficial species at the same time as predisposing host factors are required. In a recent population-based study of a Finnish population, the detection of multiple pathogenic species rather than a certain single periodontal pathogen or a pathogen combination is associated with periodontitis (Paju *et al.*, 2009). Most bacterial species associated with periodontitis are Gram-negative, obligate anaerobes; *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* form the red complex of bacteria associated with increased pocket depth and bleeding on probing (Socransky *et al.*, 1998). *Aggregatibacter actinomycetemcomitans*, associated with localised aggressive periodontitis, is a facultative anaerobe. However, some Gram-positive species, such as *Parvimonas micra* [*Peptostreptococcus micros*] (Socransky *et al.*, 1998), and viruses, such as *Epstein-Barr* virus, are also associated with this disease (Slots, 2007).

2.2.4.4. Fungal infections

Fungi are ubiquitous in the environment, and several may colonise the oral cavity. The most common fungal coloniser of the mouth is the yeast *C. albicans*, which is a harmless commensal in a large part of the population; approximately 25 % of healthy adult individuals are carriers (Percival *et al.*, 1991). Yet, there are significant differences in reported prevalence in the oral *Candida*, and age and impaired health of an individual are predisposing factors of oral *Candida* carriage (Cannon & Chaffin, 1999). Oral *Candida* carriage leads to candidiasis only when the host defence is reduced or absent. Cell-mediated immune responses and normal microbiota are important in preventing oral fungal infections, of which candidiasis is the most common. Environmental stresses described in the ecological plaque hypothesis that can cause a breakdown of the microbial homeostasis in the oral cavity include denture wearing, antibiotic treatment or low saliva flow (Marsh, 2003). These are predisposing factors for oral candidiasis. In addition to mucosal lesions, *C. albicans* in the oral cavity is also associated with dental caries, as well as periodontal and endodontic infections (Waltimo *et al.*, 1997, Järvensivu *et al.*, 2004).

2.2.4.5. Oral malodour

Oral malodour, halitosis, is not a disease but a discomfort, although some oral diseases including periodontitis may be the underlying cause. In approximately 90% of cases, the origin can be found in the oral cavity (Delanghe *et al.*, 1997). In a healthy mouth, the main cause for halitosis seems to be volatile sulphur compounds produced by Gram-negative oral bacteria on the tongue surface (Tonzetich, 1977, Hartley *et al.*, 1996). The microbial profile and the sulphur source, cysteine and methionine residues in proteins, are similar in the periodontal pockets and in the tongue coatings of patients with halitosis (Morita & Wang, 2001). Indeed, patients with periodontitis have more coatings than healthy controls on the dorsum of the tongue, measured as wet weight or volume of the bacterial coating (Morita & Wang, 2001). The microbial aetiology is not clear, but

Solobacterium moorei has recently been associated with halitosis and *Streptococcus salivarius* with health (Haraszthy *et al.*, 2007).

2.3. Probiotics in the oral cavity

2.3.1. Probiotic bacteria

The term probiotic – "for-life" – is used with different meanings, but today two main definitions are used. According to a WHO/FAO report (2002), probiotics are 'Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host'. International Life Science Institute (ILSI) Europe suggests a definition according to which a probiotic is 'a live microbial food ingredient that, when ingested in sufficient quantities, exerts health benefits on the consumer' (Ashwell, 2002). Both definitions have in common the idea that probiotic micro-organisms are living and exert proven health effects (Schrezenmeir & de Vrese, 2008).

The most commonly used probiotic bacterial strains belong to the group of lactic acid bacteria, especially lactobacilli, or to the genus *Bifidobacterium* (Saxelin *et al.*, 2005). In addition to bacteria, yeast (Buts, 2009) and even helminths are used as probiotics (Summers *et al.*, 2005). Over 100 years ago, Eli Metchnikov proposed that the use of dairy products fermented by lactic acid bacteria could have positive effects on human health (Metchnikoff, 1906). In the 1980s, the idea of improving health with beneficial bacteria became popular again, and since then the research has been intensive. *L. casei* Shirota is a probiotic strain isolated in the 1930s and used since then in fermented milk products. It is among the first probiotic strains the effects of which have been studied in controlled trials both in animal models and in humans (Yasui *et al.*, 1999, Spanhaak *et al.*, 1998). Today, the most-studied probiotic strain is *L. rhamnosus* GG, isolated at the beginning of the 1980s, and named by Professors Barry Goldin and Sherwood Gorbach (Silva *et al.*, 1987). Traditionally, probiotics have been associated with gut health, and most of the clinical interest has been focussed on the prevention or treatment of GI infections and diseases. However, during the last decade, an increasing number of established and proposed health effects for probiotic bacteria have been reported, including enhancement of the adaptive immune response, treatment or prevention of the urogenital and respiratory tract infections, or prevention or alleviation of allergies and atopic diseases in infants (Schrezenmeir & de Vrese, 2008, Saxelin *et al.*, 2005). Interestingly, probiotics are also suggested to increase the life-time of voice prosthesis by inhibiting the adhesion of unwanted microbes (Busscher *et al.*, 1997, Rodrigues *et al.*, 2004, Schwandt *et al.*, 2005).

Parvez *et al.* (2006) divide the general mechanisms of probiotics into three main categories: normalisation of the intestinal microbiota, modulation of the immune response, and metabolic effects. The effects on the intestinal microbiota can, for example, be due to competition for binding sites or secretion of antimicrobial components (Silva *et al.*, 1987, Neeser *et al.*, 2000, Pretzer *et al.*, 2005, Morita *et al.*, 2008). Protection against pathogenic microbes and an enhanced maturation of the immune system can also be due to strengthening of the mucosal barrier (Caballero-Franco *et al.*, 2007), or effects on the cytokine or chemokine production (Rautava *et al.*, 2006, Latvala *et al.*, 2008),

which in turn affect the cell-mediated and humoral immune responses. Metabolic effects can be exemplified by well-established effects, including the production of lactose-hydrolysing enzymes, or by more putative effects, including deconjugation of bile acids leading to a reduction in serum cholesterol levels (Pereira *et al.*, 2003). The mechanisms behind the probiotic action are studied intensively, and currently some of the probiotic functions are understood even at the genomic or protein level (reviewed in Lebeer *et al.*, 2008 and Ventura *et al.*, 2009). Several genes related to the adaptation of probiotic strains to the host have been identified. These include genes responsible for acid and bile tolerance, adaptation to nutritional environment and adhesion. Many mechanistic *in vitro* studies have shown that probiotic lactobacilli or their products may modulate signalling pathways related to the epithelium and immune cells. However, specific bacterial ligand-host receptor interactions remain to be established.

Probiotic effects are strain-specific, thus each individual bacterial strain needs to be tested separately, and the effects described for one strain cannot be directly applied to others. Unfortunately, mislabelling of strains in probiotic products seems to be a common problem (Yeung *et al.*, 2002, Huys *et al.*, 2006). On the other hand, multispecies or multistrain probiotic products can be even more effective than products with only one bacterial strain (Ouwehand *et al.*, 2000, Zoppi *et al.*, 2001), making the scientific evaluation of the mechanisms of the probiotic activity even a more complicated task.

2.3.2. The oral effects of probiotic bacteria

2.3.2.1. Concepts underlying the potential of oral probiotics

It is well recognised that the normal microbiota protects the oral cavity from infections, e.g. antibiotic treatment is an accepted risk factor for oral candidiasis. In addition, it has been suggested that some species of the normal oral microbiota may be associated with health benefits, while their absence is associated with diseases (Becker *et al.*, 2002, Stingu *et al.*, 2008, Aas *et al.*, 2008). For example, *S. sanguinis* is associated with health in respect to dental caries and periodontitis (Becker *et al.*, 2002, Stingu *et al.*, 2008). As far as lactobacilli are concerned, there seem to be differences in the ability of lactobacilli isolated from caries-active or healthy subjects to inhibit *S. mutans in vitro* (Simark-Mattsson *et al.*, 2007). In addition, the species composition of both *Lactobacillus* and *Bifidobacterium* microbiota is different between patients with periodontitis and periodontally healthy subjects (Hojo *et al.*, 2007a, Köll-Klais *et al.*, 2005a). Furthermore, elderly people who regularly use products containing lactic acid bacteria have lower *Candida* counts in their saliva than those who do not use such products (Hatakka *et al.*, 2007). Finally, the ecological plaque hypothesis suggests that selective pressure in environmental conditions, which can in part be generated by bacteria, for example, can change the balance between health and disease. All this suggests that it may be possible to identify probiotic bacteria which could be useful in the prevention or treatment of oral diseases. These might not necessarily be lactobacilli or bifidobacteria originally isolated to improve gut health. There are preliminary results on the safety and efficacy of a probiotic mouthwash containing oral streptococci (*S. oralis* KJ3sm, *S. uberis* KJ2sm, *S. rattus* JH145) in reducing the number of bacteria associated with dental caries and periodontitis (Zahradnik *et al.*, 2009).

The mechanisms of probiotic action in the oral cavity could be analogous to those in the intestine, including the production of antimicrobial substances (Meurman *et al.*, 1995, Nikawa *et al.*, 2004), specific competition for nutrients or growth factors (Hojo *et al.*, 2007b), inhibition of adhesion (Van Hoogmoed *et al.*, 2008) or aggregation (Kang *et al.*, 2005), which could facilitate the removal of pathogenic species from the oral cavity. However, although probiotic bacteria may influence the immune responses, the total sIgA levels in saliva seem to be unaffected by them (Kekkonen *et al.*, 2008, Paineau *et al.*, 2008). However, probiotics may modify the antimicrobial properties of the saliva by diminishing inflammation in the gingival crevice and thereby reducing the lactoferrin level in the saliva (Shimauchi *et al.*, 2008, Adonogianaki *et al.*, 1993).

2.3.2.2. Caries and caries-associated microbes

Most studies of the effects of probiotics on oral health are aimed at caries prevention, or, more precisely, at the decreasing of the number of mutans streptococci. Both probiotic *Lactobacillus* and *Bifidobacterium* strains have been used, as well as mixtures of these probiotic strains (Table 1). Several studies have shown a tendency towards decreased number of mutans streptococci in the saliva regardless of the product or strain used (Table 1). However, this effect was not seen in all studies. These discrepancies cannot be explained only by the use of different probiotic strains, as different results were obtained also using the same strains. Unfortunately, in most cases, the study groups were relatively small, and the studies were fairly short. The duration of the longest study was seven months (Näse *et al.*, 2001) and as discussed by Petti *et al.* (2001), the observed effects can, at least in some part, be an effect of the vehicle as well. For example, milk, cheese and yoghurt may well have positive effects on dental health (Petti *et al.*, 2001, Petti *et al.*, 1997, Jensen & Wefel, 1990, Ahola *et al.*, 2002). In addition, yoghurt starter strains were shown to have a slight influence on the mutans streptococcus and *Lactobacillus* levels when compared to soybean ice cream (Petti *et al.*, 2001). Thus, comparison between the base line and end number of mutans streptococci in the studies in Table 1 may lead to misconceived conclusions. In some of the studies, the effect of probiotic lactobacilli and bifidobacteria on salivary lactobacilli was studied, too. With three products, an increase in the number of salivary *Lactobacillus* was observed (Ahola *et al.*, 2002, Montalto *et al.*, 2004), but the resident and probiotic strains were not separated in detection. Even if the detected changes in the levels of oral mutans streptococci were real, the mechanisms behind them are far from clear. Possible mechanisms could include the inhibition of *S. mutans* adhesion or antimicrobial activity exhibited by the probiotic strains (Meurman *et al.*, 1995, Nikawa *et al.*, 2004, Wei *et al.*, 2002).

Lactobacilli and bifidobacteria are acidogenic, and many of the *Lactobacillus* strains in particular are also aciduric. Thus, although promising results on caries risk factors have been obtained with the probiotic bacterium *L. rhamnosus* GG (Näse *et al.*, 2001), the metabolic capacity to form acids from dietary sugars has been of concern (Hedberg *et al.*, 2008). Furthermore, results from *in vitro* studies and animal models suggest a cariogenic potential for some strains. When introduced into a salivary microcosm, probiotic *L. salivarius* W24 increased its potential cariogenicity (Pham *et al.*, 2009). In addition, *L. salivarius* LS 1952R adhered on saliva-coated hydroxyapatite and induced dental caries in rats (Matsumoto *et al.*, 2005).

Table 1. The influence of probiotic use on mutans streptococci and *Lactobacillus* counts in whole saliva or plaque samples

Strain(s)	Product (time of use)	Study group (n in probiotic group)	Effect on MS (compared to)	Effect on Lb	Follow-up	Reference
B. lactis Bb12[1]	Ice-cream (10 days)	Young adults (24)[2]	↓ (baseline)	-	ND	(Caglar *et al.*, 2008)
Bifidobacterium DN-173010[1]	Yoghurt (2 weeks)	Young adults (26)[2]	↓ (baseline)	-	ND	(Caglar *et al.*, 2005)
	Yoghurt (2 weeks)	Young adults, fixed orthodontic appliances (26)[2]	↓ (baseline)	-	MS back to baseline level in < 6 weeks	(Cildir *et al.*, 2009)
L. reuteri ATCC 55730	Yoghurt (2 weeks)	Young adults (40)[2]	↓ (placebo)	Not studied	MS counts continued to decrease (2 wk)	(Nikawa *et al.*, 2004)
	Straw (3 weeks)	Young adults (30)	↓ (baseline)	-	ND	(Caglar *et al.*, 2006)
	Tablet, allowed to dissolve in the mouth (3 weeks)	Young adults (30)	↓ (baseline)	-	ND	(Caglar *et al.*, 2006)
L. rhamnosus GG	Milk (7 months)	Children, 1 - 6 year-old (282)	- (placebo)	Not studied	ND	(Näse *et al.*, 2001)
L. rhamnosus GG and *L. rhamnosus* LC 705	Cheese (3 weeks)	Adults (41)	- (placebo)[3] ↓ (baseline)	↑ (during intervention) ↓ (after intervention)	MS counts continued to decrease (3wk)[3]	(Ahola *et al.*, 2002)
"L. sporogenes", L. bifidum, L. acidophilus, L. casei, L. rhamnosus and yoghurt starter strains	Capsules (45 days)	Adults (14)	- (baseline)	↑ (compared to baseline)	ND	(Montalto *et al.*, 2004)
	Liquid (45 days)	Adults (16)	- (baseline)	↑ (compared to baseline)	ND	(Montalto *et al.*, 2004)

MS: Mutans streptococci, Lb: Lactobacilli, ND: Not determined, [1]Strains are indistinguishable based on pulsed field electrophoresis (Grand *et al.*, 2003) [2]Cross-over design, [3]The MS counts decreased significantly in both groups (intervention and control), but after intervention only in the probiotic group.

2.3.2.3. Periodontal diseases

The first studies of the use of probiotics for enhancing oral health were for the treatment of periodontal inflammation (Kragen, 1954). Patients with different periodontal diseases, gingivitis, periodontitis and pregnancy gingivitis, were locally treated with a culture supernatant of a *L. acidophilus* strain. Significant recovery was reported for almost every patient. Recently, probiotic treatment with two *L. reuteri* strains enhanced the effects of oral hygiene instructions in improving the gingival health as measured by decreased gum bleeding (Krasse *et al.*, 2006, Twetman *et al.*, 2009). In the first study, two unspecified *L. reuteri* strains were administered in tablets, in the second *L. reuteri* ATCC 55730 and ATCC PTA 5289 were given in chewing gum. The use of this probiotic chewing gum also decreased the levels of pro-inflammatory cytokines in GCF (Twetman *et al.*, 2009). In another study, gingival pocket depth, particularly in high risk groups, such as smokers, was decreased when *L. salivarius* WB21 was used for 8 weeks with no reported change in oral hygiene behaviour (Shimauchi *et al.*, 2008). In a separate study, the use of *L. salivarius* WB21 also affected the number of periodontopathogens in plaque (Mayanagi *et al.*, 2009).

Supporting the clinical treatment of periodontitis and gingivitis seems to be a potential target for probiotic lactic acid bacteria or bifidobacteria. The inflammation in these conditions is alkaline, thus the milieu could be changed by acid-producing strains. In addition, animal experiments support the idea of using beneficial bacteria in directing the normalisation of the microbial flora (Nackaerts *et al.*, 2008). However, as discussed above, the acidogenicity of the strains can not be overlooked. *L. salivarius* strain WB21 has been used for decreasing gingival inflammation, but some other strains of this species are able to make a biofilm model more cariogenic and induce caries in rats (Matsumoto *et al.*, 2005, Pham *et al.* 2009).

2.3.2.4. Other applications

Despite the promising results of mouse experiments in treatment of oral *Candida* (Elahi *et al.*, 2005), there are only two studies in which the effects of probiotic bacteria on oral *Candida* infection in humans have been investigated (Hatakka *et al.*, 2007, Ahola *et al.*, 2002). When a test group of elderly people consumed cheese containing *L. rhamnosus* strains GG and LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS for 16 weeks, the number of high oral yeast counts decreased, but no changes were observed in mucosal lesions (Hatakka *et al.*, 2007). In a shorter study with younger subjects, no significant difference was observed between the effects of the probiotic and control cheese on salivary *Candida* counts (Ahola *et al.*, 2002).

The treatment of halitosis with probiotics has also been studied. Successful reduction in the concentration of volatile sulphur compounds in the exhaled breath has been observed with *S. salivarius* K12, three *Weissella confusa* isolates and with a lactic acid-forming bacterial mixture, not specified by the authors of that work (Burton *et al.*, 2006, Kang *et al.*, 2006b, Horz *et al.*, 2007, Iwanicka-Grzegorek *et al.*, 2005). The first report of the treatment of halitosis with a probiotic bacterium was the treatment of gut-associated halitosis (Henker *et al.*, 2001). In that case report, a 9½-year-old girl was treated with *E. coli* Nisle 1917 for almost three months. The halitosis was cured for at least four years.

2.3.3. Colonisation of the oral cavity by probiotic bacteria

Most products containing probiotic bacteria are consumed orally, and the bacteria in them are thus in contact with the oral surfaces unless administered in capsules. Furthermore, lactobacilli and bifidobacteria form a part of the resident microbiota of the oral cavity. The same *Lactobacillus* species colonise the oral cavity and the colon (Ahrne *et al.*, 1998, Maukonen *et al.*, 2008), but, although bifidobacterial species are a part of the oral microbiota, those found in the mouth and in the intestine are mostly different (Rotimi & Duerden, 1981, Maukonen *et al.*, 2008, Beighton *et al.*, 2008). Information on the oral colonisation of commercially used probiotic bacteria is scarce and the results controversial.

L. rhamnosus GG and two different *L. reuteri* strains (ATCC 55730 and one not specified by the authors of the article in question) have been reported to colonise the oral cavity of 48 – 100% of the volunteers consuming products containing them (Yli-Knuuttila *et al.*, 2006, Caglar *et al.*, 2009, Krasse *et al.*, 2006, Meurman *et al.*, 1994). Furthermore, consumption of a mixture of seven different *Lactobacillus* strains increased the number of salivary *Lactobacillus* counts, although the identities of the strains in the saliva were not determined (Montalto *et al.*, 2004). In contrast, *L. rhamnosus* GG could not be detected in the saliva of any of the volunteers consuming yoghurt containing it (Busscher *et al.*, 1999). Similarly, Maukonen *et al.* (2008) did not detect any of the probiotic bacteria administered in capsules in saliva samples. *L. reuteri* ATCC 55730 (= *L. reuteri* SD2112) does not seem to influence the total number of salivary lactobacilli (Caglar *et al.*, 2006), but *L. rhamnosus* GG may increase it (Ahola *et al.*, 2002). It has also been suggested that introduced probiotic bacteria may usurp space from resident lactobacilli (Ahola *et al.*, 2002, Krasse *et al.*, 2006). Of the probiotic bifidobacteria, only the presence of *B. animalis* subsp. *lactis* Bb12 (*B. lactis* Bb12) in oral samples has been studied, but it has not been detected in the mouth after the consumption of products containing it (Maukonen *et al.*, 2008, Taipale *et al.*, 2007, Eva Söderling, personal communication). *S. salivarius* K12, used for treating oral malodour, temporarily colonises the oral cavity for a short time after use (Horz *et al.*, 2007).

Overall, some probiotic *Lactobacillus* and *Streptococcus* strains seem to be able to temporarily colonise the oral cavity of some people. Probiotic bacteria are often ingested during antibiotic treatment to prevent possible side effects. In addition, according to the instructions for the only commercial probiotic product for oral health purposes in Finland, it is recommended to be used after brushing the teeth. In both cases, the probiotic bacteria are most likely to colonise differently in the presence and in the absence of the normal microbiota. Unfortunately, there are no data available on the colonisation of probiotic bacteria in these cases.

2.4. Selected genera and species of special interest for this study

2.4.1. Genus Lactobacillus

The genus *Lactobacillus* is a large, heterogeneous group of lactic acid bacteria (Axelsson, 2004). Lactobacilli are usually facultative or microaerophilic, lack catalase and grow

optimally under slightly acidic conditions. The lactic acid bacteria are divided into three groups according to their sugar fermentation patterns and *Lactobacillus* species belong to all these three groups. Obligate homofermentative bacteria ferment sugars by glycolysis and thus produce lactate, whereas the obligate heterofermentative ones use only the 6-phosphogluconate / phosphoketolase pathway, in which the end products are lactate and ethanol. The facultatively heterofermentative species fall between these two categories and are homofermentative concerning hexoses and heterofermentative concerning pentoses (Axelsson, 2004). Previously, the obligate homofermentative group included species now classified to *Weissella*; some of which have now been suggested to have potential as oral probiotics (Kang *et al.*, 2005, Kang *et al.*, 2006b, Kang *et al.*, 2006a).

Lactobacilli are widely spread in nature. In humans, they are a part of the normal microbiota of the oral cavity, gastrointestinal tract and vagina. No species specific to the oral cavity are known, as the same species seem to colonise both ends of the gastrointestinal tract (Ahrne *et al.*, 1998, Maukonen *et al.*, 2008). In the oral cavity, lactobacilli usually comprise less than 1% of the total cultivable microbiota. Commonly isolated species include *L. paracasei, L. plantarum L. rhamnosus* and *L. salivarius,* for example (Simark-Mattsson *et al.*, 2007, Ahrne *et al.*, 1998, Maukonen *et al.*, 2008, Colloca *et al.*, 2000). Normally, lactobacilli are regarded as a part of the normal oral microbiota, but contradictory opinions claiming that caries-associated lactobacilli are exogenous and opportunistic colonisers which are possibly acquired from food have also been presented (Caufield *et al.*, 2007). On the other hand, results obtained from studies on caries-preventive measures suggest that transmission of lactobacilli from mothers to children possibly via saliva is likely (Köhler & Andreen, 1994). However, there are no conclusive results on how oral lactobacilli are acquired.

Lactobacilli are not usually pathogenic, but as the most acidogenic among the lactic acid bacteria they are associated with the progression of dental caries. Indeed, the number of lactobacilli in saliva is sometimes used as a part of caries risk estimation (Larmas, 1992). No species associated with caries are known, but there can be differences in *Lactobacillus* microbiota of caries-active and healthy subjects (Simark-Mattsson *et al.*, 2007). However, the species identification of lactobacilli with the API fermentation test used by Simark-Mattsson *et al.* (2007) has recently been shown to be imprecise (Teanpaisan & Dahlen, 2006). Recent evidence also suggests that different *Lactobacillus* species might be related to gingival health and disease (Kõll-Klais *et al.*, 2005a, 2005b), obligately homofermentative species, particularly *L. gasseri*, being associated with health. However, not all studies have found such an association (Hojo *et al.*, 2007a).

2.4.2. Genus Bifidobacterium

Bifidobacteria are Gram-positive, non-motile, non-sporulating bacterial species which commonly have a Y-shaped morphology, although the morphology is dependent on the culture medium. Bifidobacteria are strictly anaerobic, but their degree of oxygen tolerance depends on the species or even the strain and culture media used (Ballongue, 2004). The level of acid tolerance is also strain specific (Mättö *et al.*, 2004). Bifidobacteria contribute to human well-being; they participate in digestion and are suggested to play

a role in the maturation of the developing immune system. In addition, they produce significant amounts of vitamins which contribute to host nutrition (Ballongue, 2004).

Although bifidobacteria have been traditionally associated with intestinal microbiota, they also seem to be a part of the oral commensal microbiota. According to early culture-based studies, bifidobacteria are among the first anaerobes in the oral cavity (Rotimi & Duerden, 1981). More recent data are not available concerning the colonisation process of the oral cavity by bifidobacteria. It has been suggested that oral bifidobacteria could also be acquired from probiotic food (Beighton *et al.*, 2008). Thus, how oral bifidobacteria are acquired is unknown. Of the *Bifidobacterium* species, nine are isolated from humans (Ballongue, 2004); of these *B. bifidum, B. dentium* and *B. longum* are found in oral samples (Crociani *et al.*, 1996, Maukonen *et al.*, 2008, Beighton *et al.*, 2008).

Bifidobacteria are acidogenic but, in general, they are not as acid-tolerant as lactobacilli, for example, as demonstrated by studies related to fermented foods, and the survival and growth of bacteria in the intestine (Graeme *et al.*, 2005, Ongol *et al.*, 2007). However, there are significant differences between the species and strains (Crittenden *et al.*, 2001, Collado & Sanz, 2006*). B. animalis* (*B. lactis*) is significantly more acid-tolerant than common human intestinal bifidobacterial species (Crittenden *et al.*, 2001). Interestingly, human oral isolates are both acidogenic and aciduric (van Houte *et al.*, 1996). However, van Houte *et al.* (1996) also included other members of family *Bifidobacteriaceae* in their study and did not separate between genera; thus no conclusive statements on the aciduric nature of oral bifidobacteria can be made based on their results. However, their findings are in accordance with the fact that oral bifidobacteria are associated with the progression and severity of dental caries (Becker *et al.*, 2002, Mantzourani *et al.*, 2009). In addition, *B. longum* and *B. breve* have been detected in infected root canals (Chavez de Paz *et al.*, 2004). Furthermore, similar intraoral factors that favour traditionally caries-associated micro-organisms, may favour bifidobacteria, too (Beighton *et al.*, 2008). On the other hand, some *Bifidobacterium species* may be associated with periodontal health (Hojo *et al.*, 2007a).

2.4.3. Helicobacter pylori

Helicobacter pylori is a Gram-negative, microaerophilic, motile, non-sporing, curved or spiral rod causing gastritis and peptic ulcer disease, and is a significant risk factor for gastric cancer. However, the majority of infected subjects are asymptomatic (Amieva & El-Omar, 2008). It has been estimated that 50% of the world's population is infected with *H. pylori*. The infection is more common in developing countries. In Finland, approximately 30 – 40% of the population is infected, the infection being less common in children than in older people (Färkkilä, 2009). The infection is usually acquired in childhood, and it usually persists, if not treated. Despite intensive studies, the exact mode of *H. pylori* transmission is not known. The transmission seems to need close contact (Amieva & El-Omar, 2008). The main hypotheses for the transmission are oral-oral, gastric-oral or faecal-oral and, most probably, the main transmission modes are different in different populations (Amieva & El-Omar, 2008, Delport & van der Merwe, 2007).

It has been suggested that the oral cavity might serve as an extra-gastric reservoir for *H. pylori*. *H. pylori* adheres to salivary agglutinin gp340 and salivary mucins (Prakobphol *et al.*, 2000, Linden *et al.*, 2008), and also co-aggregates with oral bacteria (Ishihara *et al.*, 1997, Andersen *et al.*, 1998); it thus has a means to adhere to the oral cavity. In addition, a few years after the isolation of *H. pylori* from gastric biopsies, the bacterium was also isolated from an oral sample (Krajden *et al.*, 1989), but despite several attempts, only very few studies have subsequently reported having isolated *H. pylori* (Khandaker *et al.*, 1993, Ferguson *et al.*, 1993, Parsonnet *et al.*, 1999). With other methods, including PCR, the Gram stain and urease test, the detection rate of *H. pylori* in saliva, dental plaque or oral swab samples varies between 0 – 100% (Leimola-Virtanen *et al.*, 1995, Gebara *et al.*, 2004, Olivier *et al.*, 2006, Anand *et al.*, 2006, Miyabayashi *et al.*, 2000, Luman *et al.*, 1996, Liu *et al.*, 2009, Burgers *et al.*, 2008). There seem to be populational differences but the discrepancies can be partly explained by the different methodologies used. In the earliest studies, *H. pylori* was detected with a urease test or tests based on bacterial morphology (Ozdemir *et al.*, 2001, Young *et al.*, 2001); thus, the possibility for false positives in oral samples was high. Whether *H. pylori* really colonises the oral cavity is questionable. On the one hand, its detection may be related to gastric reflux, but some considered it to be a part of the normal oral microbiota (Song *et al.*, 2000).

The importance of oral *H. pylori* can be debated. Certainly, the oral cavity is the portal of entry (Marshall *et al.*, 1985). There is some evidence, that oral *H. pylori* could re-colonise patients after eradication of the bacterium from the stomach (Miyabayashi *et al.*, 2000, Avcu *et al.*, 2001). After *H. pylori* was detected in oral samples, the bacterium has been associated with several oral diseases, including oral aphthous ulcers, burning mouth syndrome and even caries (Birek *et al.*, 1999, Gall-Troselj *et al.*, 2001, Liu *et al.*, 2008). The idea of *H. pylori* causing oral ulcers is tempting but not proven. In some cases, the observed association between oral manifestations and *H. pylori* can be explained by factors other than oral *H. pylori*. For example, iron deficiency anaemia, known to have oral manifestations, has been associated with *H. pylori* (Nakata & Ichinose, 2003).

3. AIMS OF THE STUDY

As probiotic products are widely used and at least some of the bacterial strains in them seem to be able to transiently colonise the oral cavity, it is important to understand their effects in the oral environment. The most commonly used probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium*, the genera that are also associated with dental caries. Thus, the safety of the probiotic bacteria also needs to be evaluated with respect to dental health. On the other hand, understanding how probiotic bacteria behave in the oral cavity may bring a means to control pathogenic bacteria in the mouth. The aim of this study was to evaluate *in vitro* the oral effects of probiotic bacteria already in commercial use.

The specific aims were:

1) To compare the oral colonisation potential of probiotic lactobacilli and bifidobacteria *in vitro* by studying their survival in saliva and their adhesion to oral surfaces.

2) To estimate *in vitro* the cariogenic potential of probiotic lactobacilli and bifidobacteria based on their acid production.

3) To estimate the effects of probiotic bacteria on the oral microbial ecology by studying their effects on *in vitro* pellicle and the adhesion of other bacteria, and their capability to activate the antimicrobial peroxidase system. An aim was also to study the effects of the activated peroxidase system on the probiotic bacteria themselves and on other bacteria.

The hypothesis was that, analogically to the intestine, probiotic bacteria could adhere to the oral surfaces and modify the oral microflora by competing for binding receptors on the oral surfaces or by modifying the pellicle containing the receptor proteins. Moreover, hydrogen peroxide-producing lactobacilli could activate the antimicrobial peroxidase system and thereby inhibit their own metabolic activity and the viability of other bacteria.

4. MATERIALS AND METHODS

A summary of materials and methods used in this study is presented below. Detailed information can be found in **I-V**.

4.1. Bacteria and their growth conditions

Lactobacilli and bifidobacteria (I-V)

This study included 16 *Lactobacillus* and 8 *Bifidobacterium* strains with known probiotic properties (10 strains), common dairy strains (6 strains), faecal isolates with potential probiotic characteristics from healthy infants and adults (7 strains) and one strain from a culture collection originally isolated from saliva and with no known probiotic properties (Table 2). The faecal isolates were originally identified down to the species or strain level based on their sugar fermentation pattern and by protein patterns if required (Apostolou *et al.*, 2001, He *et al.*, 2001a, He *et al.*, 2001b). Two of the commercial bifidobacteria (*B. animalis* subsp. *lactis* and *Bifidobacterium* species 420) were likely to be same strain (Grand *et al.*, 2003, labelled in Table 2), but as the strain identification was not included in this study, they are treated as separate strains. In addition, two faecal *L. rhamnosus* isolates indistinguishable from *L. rhamnosus* GG (labelled in Table 2) based on pulsed-field gel electrophoresis (PFGE) analyses were included in the study (I).

Lactobacillus strains were grown in MRS broth (de Man, Rogosa and Sharpe, Difco™, Difco laboratories, Sparks, MD, USA) or on MRS or M17 (Oxoid) agar in an anaerobic atmosphere (10% CO_2, 80% N_2, 10% H_2) (I, adhesion experiments on hydroxyapatite, survival experiments), in air with 5% CO_2 (I, adhesion experiments on microtitre wells) or in atmospheric air (II, III, V). *Bifidobacterium* strains were grown in RCM (Reinforced Clostridial Medium, LabM, Lancashire, UK) in an anaerobic atmosphere.

Table 2. Lactobacillus and Bifidobacterium strains used I-V

Original publication	Strain		Origin
	Lactobacillus		
I, II	*L. acidophilus* NFCM	Probiotic	Danisco, USA
III	*L. casei*	Saliva	ATCC 11578
I, II	*L. casei* 921	Dairy	ATCC 334
I, II, III	*L. casei* Shirota	Probiotic	Yakult®, Yakult Honsha, Japan
I*, II	*L. delbrueckii* sp. *bulgaricus* 365	Dairy	ATCC 11842
I, II	*L. johnsonii* LA1	Probiotic	LC1®, Nestlé Ltd, Switzerland
I*	*L. paracasei* 12.11a	Faecal	(Apostolou *et al.*, 2001)
I	*L. paracasei* 8.12a	Faecal	(Apostolou *et al.*, 2001)
I	*L. paracasei* 8.16b	Faecal	(Apostolou *et al.*, 2001)
I, II	*L. paracasei* F19	Probiotic	Arla Ltd, Sweden
I, II, V	*L. plantarum* 299v	Probiotic	DSM 9843, ProViva, Probi AB, Sweden
I, II, III, V	*L. reuteri* SD 2112	Probiotic	Rela®, Ingman Foods, Finland (ATCC 55730)
I	*L. rhamnosus* 11.4a	Faecal	(Apostolou *et al.*, 2001)
I*	*L. rhamnosus* 5.1a	Faecal[1]	(Apostolou *et al.*, 2001)
I	*L. rhamnosus* 5.3a	Faecal[1]	(Apostolou *et al.*, 2001)
I	*L. rhamnosus* 5.5a	Faecal	(Apostolou *et al.*, 2001)
I, II, III, V	*L. rhamnosus* GG	Probiotic	ATCC 53103
I, II	*L. rhamnosus* LC 705	Dairy	Valio Ltd, Finland
	Bifidobacterium		
I	*B. adolescentis* A 16	Faecal	(He *et al.*, 2001b)
I*	*B. infantis* A3	Faecal	(He *et al.*, 2001b)
I, II, III	*B. animalis* subsp. *lactis*[2] (*B. lactis* Bb12)	Probiotic	Chr. Hansen, Denmark
I	*B. longum* 2C	Probiotic	(Makelainen *et al.*, 2003)
I	*B. longum* 46	Probiotic	(Makelainen *et al.*, 2003)
II	*B. longum* 913	Dairy	Wisby, Niebüll, Germany[3]
II	*Bifidobacterium* sp. 1100	Dairy	Wisby, Niebüll, Germany[3]
II	*Bifidobacterium* sp. 420[2]	Dairy	Wisby, Niebüll, Germany[3]

1 Indistinguishable from L. rhamnosus GG based on pulsed-field gel electrophoresis (I)
2 Indistinguishable from each other based on pulsed-field gel electrophoresis (Grand *et al.*, 2003)
3 Now: Danisco, Niebüll, Germany
* These strains were selected for further experiments in I and III (see p. 47, I and III for details).

Streptococci (II, III, V) and lactococci (I)

S. mutans strains Ingbritt and MT 8148, as well as *S. gordonii* DL1 were grown in the BHI broth (Brain Hearth Infusion, Difco laboratories, Sparks, MD, USA) at 37ºC. For adhesion experiments, the streptococci were grown first overnight and then three to four hours into the mid logarithmic phase. *Lactococcus* (*Lc.*) *lactis* MG 1363 was grown in MRS broth at 30ºC.

***Fusobacterium nucleatum* (I) and *Aggregatibacter actinomycetemcomitans* (V)**

F. nucleatum ATCC 10953 was cultivated on Brucella agar (Difco laboratories, Sparks, MD, USA) supplemented with 70ml horse blood per litre in an anaerobic atmosphere (10% CO_2, 80% N_2, 10% H_2).

A. actinomycetemcomitans ATCC 29523 was cultivated in the BHI broth or on blood agar (Blood agar Base No. 2, Difco, Becton-Dickinson, Sparks, MD, USA supplemented with 5% of sheep blood) in candle jars.

***Helicobacter pylori* (IV, V)**

Helicobacter pylori ATCC 43504 (IV, V) and five clinical *H. pylori* isolates (IV) were used. The bacteria were cultured on the Brucella agar (Difco laboratories, Sparks, MD, USA) supplemented with 7% (v/v) of horse blood and, in study IV, also with 1% (v/v) IsoVitalexsupplement (BBL, Becton-Dickinson, Sparks, MD, USA) in a microaerophilic atmosphere generated with Anaerocult C (Merck, Darmstadt, Germany).

4.2. Buffers and salt solutions

The following buffers and salt solutions were used. The solutions were made in distilled water and filter-sterilised, when needed (II, IV, V).

- Buffered KCl (I, III)

50.0 mM KCl, 0.35 mM K_2HPO_4, 0.65 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ at pH 6.5 (Clark *et al.*, 1978)

- Fermentation minimal medium (FMM, II, V)

50 mM KCl, 5 mM NaCl, 2 mM $MgSO_4$, 2 mM $MnCl_2$, 8 mM $(NH_4)_2SO_4$, 1.5 µM thiamine and 8 µM niacin at pH 7.0 (Dashper & Reynolds, 1990)

- Solution I (IV, V)

9 mM Na_2HPO_4, 24 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 67 mM Na_2SO_4 at pH 6.5 and 5.0. The buffer was adjusted to pH 5.0 by adding 0.1 M acetic acid. (Modified from Klebanoff & Clark (1975) by Ihalin *et al.* (1998).

- Phosphate buffered saline (I), PBS (Gibco™, Invitrogen, Paisley, Scotland, UK)

- HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, Sigma, St. Louis, USA) buffered Hank's balanced salt solution (I)

4.3. Saliva

The parotid saliva used in adhesion experiments (I, III) was collected on ice by means of Lashley cups and stimulation by a Salivin® lozenge (Pharmacia Ltd, Vantaa, Finland). Unstimulated whole saliva (I, III) and paraffin-stimulated whole saliva (I, IV) were

clarified by centrifugation and, when required, sterilised either by UV-radiation (254 nm) (I) or filtering (0.45 μm) (IV). The salivas were collected either each morning before the experiment or pooled, clarified by centrifugation and stored in aliquots in a freezer. There is always a choice to be made regarding how saliva is used in an experimental model. Some of the salivary components, including some protein aggregates and proteins attached to bacterial and human cells, are always lost when the saliva is clarified by centrifugation. Parotid saliva collected directly from ducts has the advantage that it needs neither clarification nor sterilisation, but it lacks many of the components found in the whole saliva. UV radiation, as well as the freezing and thawing steps may influence the conformation of salivary proteins. On the other hand, day-to-day variation of saliva can be avoided when the same saliva or saliva pool is used in different experiments.

The volunteers were asked to avoid smoking, eating, drinking and use of oral hygiene products for one hour before saliva collection. As the sensitivity to the activated peroxidase system was tested, the salivas of non-smoking volunteers were used (I, IV).

Informed consent was obtained from volunteers donating saliva. Permission to collect saliva samples was granted by the Joint Ethical Committee of the Turku University and the Turku University Central Hospital.

4.4. Chemical assays

Peroxidase enzyme, peroxidase activity and hypothiocyanate (HOSCN/$OSCN^-$) (II, IV, V)

Bovine lactoperoxidase (LPO, Sigma Aldrich, St. Louis, MO, USA) was used in all experiments in which purified peroxidase was required (II, IV, V). Human SPO and bovine LPO resemble each other both structurally and catalytically (Månsson-Rahemtulla *et al.*, 1988). Bovine LPO is therefore widely used in studies of the peroxidase-mediated functions of human saliva.

The peroxidase activity was measured at RT by following the rate of oxidation of the coloured (5,5)-dithiobis-2-nitrobenzoic acid (Nbs) to colourless $(Nbs)_2$ by $OSCN^-$ ions generated during the oxidation of SCN^- by peroxidases in the saliva or by the bovine lactoperoxidase (assays in buffer) (Wever *et al.*, 1982, Månsson-Rahemtulla *et al.*, 1986). The amount of HOSCN/$OSCN^-$ was quantified by a reaction with Nbs as described by Pruitt *et al.* (1983) and Aune & Thomas (1977). All oxidised SCN^- was determined as HOSCN/$OSCN^-$, although other oxidation products are generated in the peroxidase-catalysed reactions between KSCN and H_2O_2, too (reviewed in Davies *et al.*, 2008).

Lysozyme (IV)

The lysozyme activity in the saliva was quantified by measuring the decrease of absorbance at 540 nm of a commercial nonviable *Micrococcus lysodeikticus* culture (Bacto lysozyme substrate, Difco) in 66 mM Na_2HPO_4 and NaH_2PO_4, pH 6.2, using the hen egg white lysozyme as a standard (Difco manual, 1984).

Thiocyanate (IV)

The salivary SCN^- concentration was measured by allowing ferric nitrate to react with salivary SCN^-, the amount of reaction product was then measured spectrophotometrically using a KSCN standard (Betts & Dainton, 1953).

Proteins (IV)

The protein concentration in the saliva was measured by a colorimetric method using the Folin phenol reagent (Lowry *et al.*, 1951).

Hydrogen peroxide (V)

Detection of H_2O_2 was based on measuring the formation of a coloured product in a horseradish peroxidase (HRP)-catalysed reaction between H_2O_2 and tetramethyl benzidine (TMB, Sigma Aldrich, Steinheim, Germany). Initial testing for H_2O_2 was performed on agar plates (Rabe & Hillier, 2003). The quantitative method was modified from Josephy *et al.* (1982). The quantitative results were ascertained by using *o*-diadisidine (3,3'-dimethoxybenzidine, Sigma-Aldrich, Steinheim, Germany) as a substrate for HRP (Juárez Tomás *et al.*, 2004).

4.5. Survival of bacteria in saliva (I, IV)

Survival of lactobacilli and bifidobacteria in saliva (I)

Pooled, sterilised and stimulated whole saliva was used in the experiments. The bacteria were incubated in the saliva at 37°C for 24 h, the bifidobacteria in anaerobic conditions. In a pilot study, the optical density at 492 nm ($OD_{492\ nm}$) was measured at two-hour intervals for 24 h. Because $OD_{492\ nm}$ did not increase during the incubation period, it was assumed that there was no growth and the OD_{492} nm was measured only at the beginning of the incubation and after 24 h. Samples were taken before and after the 24-hour incubation, diluted and plated, and the colonies were counted after two days of incubation.

Effects of the activated peroxidase system on* Lactobacillus rhamnosus *GG,* Bifidobacterium lactis *Bb12 and* Helicobacter pylori *ATCC 43504 in saliva (I, IV)

Pooled saliva samples of stimulated whole saliva, stored in a freezer, were used for testing the effects of the salivary peroxidase system on *L. rhamnosus* GG and *H. pylori* ATCC 43504. The activity against *B. lactis* Bb12 was tested in fresh saliva. As the lysozyme activity was completely lost during the sterilisation and freezing steps (IV), a physiological concentration of lysozyme was added to the saliva in the experiments with *H. pylori*.

L. rhamnosus GG and *B. lactis* Bb12 were harvested from overnight cultures by centrifugation, washed and resuspended in PBS, and the OD_{600} was adjusted to 5.0 (≈ 10^9 CFU/ml). An aliquot of 100 µl of the bacterial suspension was added to 850 µl of saliva supplemented with 50 µl of PBS or 1 mM H_2O_2, giving a final concentration of 50 µM H_2O_2. After a 1-hour incubation at 37°C, aliquots of 100 µl were taken, diluted and plated. The colonies were counted after two days of incubation.

The effects of the saliva and saliva supplemented with H_2O_2, lysozyme, or both on *H. pylori* ATCC 43504 were investigated at pH 7.1 and 5.0. 100 µl of bacterial suspension (approx. 10^9 CFU/ml) was added to 800 µl of sterilized saliva supplemented with 100 µl of buffer, lysozyme, H_2O_2, or both lysozyme and H_2O_2 (final concentrations 100 µM H_2O_2 and 50 µg lysozyme/ml). After a 1-hour incubation at 37°C, dithiothreitol (DTT) was added, and aliquots of 100 µl were removed, diluted and plated. The colonies were counted after three days of incubation. The pH of the assays was measured before and after incubation.

4.6. Adhesion experiments (I, III)

Two commonly used adhesion assays, adhesion on microtitre wells (MTW; I) and adhesion on hydroxyapatite (HA; I, III) were used.

Binding of bacteria on saliva-coated microtitre wells (I)

The bacteria were labelled by adding 10 µCi/ml of methyl-1,2[^{3}H] thymidine (Amersham, UK) in the growth medium. Bacteria from overnight cultures were harvested by centrifugation and washed with PBS. The salivary proteins were passively immobilised to polystyrene Maxisorp microtitre plate wells (Nunc, Roskilde, Denmark) by incubating saliva on plates. The adhesion experiment was performed as described above (Kirjavainen *et al.*, 1998). Briefly, labelled bacteria were added to the wells and allowed to adhere for 60 minutes. After washing with PBS to remove the unbound bacteria, the adhered bacteria were released and lysed, and the amount of bound bacteria was measured with a scintillation counter. Binding was expressed as the percentage of bound bacteria from the added bacteria.

Binding of bacteria on saliva or bovine serum albumin-coated hydroxyapatite (I, III)

The basis for all the adhesion experiments was the same as described above for the *Streptococcus* species (Clark *et al.*, 1978, Gibbons & Etherden, 1985). Briefly, the bacteria were labelled by adding ^{35}S-methionine (10 or 20 µCi/ml, Amersham Biosciences, UK) in the growth medium. After growth, the bacteria were washed and resuspended in buffered KCl. HA beads (5 mg, BDH Chemicals Ltd, Poole, UK) were coated with saliva for 60 minutes at RT. In study I, the possibly existing free HA surfaces were blocked with BSA. Bacterial suspension with labelled bacteria was added, and the bacteria were left to adhere for 60 minutes, after which the unbound bacteria were washed away and the amount of bound bacteria was measured with a scintillation counter. Binding was expressed as the percentage of bound bacteria from the added bacteria.

Binding of* Lactobacillus *and* Bifidobacterium *species on saliva and* Fusobacterium nucleatum *or* Streptococcus mutans *or* gordonii*-coated hydroxyapatite (I, III)

F. nucleatum ATCC 10953 was labelled by growing bacteria on Brucella agar in the presence of ^{35}S-methionine (100 µCi), and the binding of *F. nucleatum* to saliva-coated HA was tested with the labelled *F. nucleatum* suspension. The binding of *Lactobacillus* and *Bifidobacterium* strains was tested as described above with the following differences: After coating HA beads with saliva (or buffered KCl), the beads were incubated for 1 h

in a suspension made of unlabelled *F. nucleatum* or streptococci. The beads were washed and incubated for an additional 15 minutes in saliva. After washes, the *Lactobacillus* or *Bifidobacterium* suspension was added over the *F. nucleatum* or streptococci. After a 1-hour incubation, the beads were washed and the amount of bound bacteria was calculated. Binding was expressed as the percentage of bound bacteria from the added bacteria.

Binding of streptococci on pellicles made of parotid saliva treated with lactobacilli or bifidobacteria (III)

Parotid saliva was incubated for 60 or 180 minutes with buffered KCl (1:3) or with bacterial suspensions made of probiotic bacteria or lactobacilli used as a control (10^8 CFU/ml or 2 x 10^9 CFU/ml). The bacteria were then removed and the supernatant was used to coat HA. Control pellicles for the adhesion experiments were made by first coating HA with parotid saliva and then with a supernatant of a bacterial suspension in a buffer. The adhesion experiments using these modified pellicles were performed as the adhesion experiments described above.

Adherence of* Lactobacillus *and* Bifidobacterium *species on buccal epithelial cells (I)

The assay was made as described previously (Johansson *et al.*, 2000), the steps being as follows: Buccal epithelial cells were collected from one healthy female volunteer. The cells were washed and resuspended in buffered KCl to give an $OD_{600\ nm}$ of 0.500. Bacterial suspensions (appr. $2 \cdot 10^7$ CFU/ml) prepared in buffered KCl from overnight cultured bacteria and equal amounts of buccal epithelial cells were incubated for 60 minutes. Control cells were treated only with buffer. After washing the cells, those with attached bacteria were stained with crystal violet. All bacteria (both indigenous and the added lactobacilli or bifidobacteria) bound to the buccal epithelial cells were counted using a light microscope. From each sample 30 cells were counted.

4.7. Aggregation experiments (III)

Washed bacteria were resuspended in buffered KCl with 10^9 CFU/ml. Each of the bacterial suspensions was mixed 1:1 with parotid saliva or with buffered KCl and the formation of visible aggregates was followed for 2 minutes.

4.8. Production of acids (II, V)

Acid production from different sugars (glucose, sucrose, lactose) and sugar alcohols (xylitol, sorbitol) by lactobacilli or bifidobacteria was followed in FMM (composition on p. 37) by recording the pH electrometrically. To describe the procedure briefly, the bacteria, grown overnight, were washed, and a bacterial suspension of 10^8 CFU/ml was prepared in FMM. The suspension was divided into 1 ml aliquots. Before the addition of the sugars, sugar alcohols or water (control), the bacteria were depleted of endogenous reserves of carbohydrates by incubating them at 37ºC in a water bath without agitation

for 20 minutes. After the pre-incubation, the pH was monitored (time 0) and 100 µl of 100 mM sugar, sugar alcohol or water (control) was added. The pH was followed at 5, 10, 15 and 30 minutes after addition of the sugars.

To test the effects of the components of the LPO system (V) on the generation of acids from glucose, these components of the LPO system, all together or individually, were added (final concentrations being 5µg/ml of LPO, 1mM of KSCN and 50 µM of H_2O_2) to the bacterial suspension.

4.9. Binding of salivary proteins by lactobacilli and bifidobacteria (III, V)

Effects of the probiotic strains on the on the salivary pellicle protein composition (III)

Effects on protein composition

Experimental pellicles were prepared by coating 100 mg of HA for 60 minutes with 1.25 ml of the parotid saliva first incubated for 180 minutes with the probiotic bacteria, control strains or a buffer (control). After three washes, the proteins were released by vigorous vortexing in 1 ml of 0.1% SDS, and the samples were concentrated by freeze drying. The proteins were resuspended in the SDS sample buffer with a reduced amount of SDS and no reducing agent. To analyse the protein composition of the pellicles, SDS polyacryl amide gel electrophoresis (SDS-PAGE) was performed in a BioRad Mini-protean II cell using 4 – 15% gradient Tris-HCl gels (BioRad Laboratories, Hercules, CA, USA). The gels were stained with a silver stain.

Protein identification

The presence (and removal) of salivary agglutinin gp340 in the pellicles was ascertained with the Western analysis (see below) and the salivary peroxidase was identified by the proteomics service at the Turku Centre for Biotechnology (Turku, Finland).

Binding of salivary agglutinin gp340 and lactoperoxidase by bacteria (III, V)

The binding of agglutinin to bacterial cells was tested by slightly modifying the method described by Prakobphol *et al.* in 2000. In brief, bacterial suspensions of the probiotic or control strains were incubated with an equal volume of parotid saliva diluted 1:1 in buffered KCl. The binding took place at RT for 0, 15, 30 or 60 minutes.

LPO was labelled with EZ-Link® Sulfo-NHS-LC biotin (Pierce, Rockford; IL, USA). To measure the binding of LPO on lactobacilli in FMM, washed bacteria were incubated with 5 µg/ml of labelled LPO for 30 minutes. In MRS, the binding was detected after 6 hours of growth.

In all experiments, the cells were pelleted by centrifugation for 10 minutes, a sample was taken from the supernatant, and the pellet was washed. To release the bound proteins, the bacterial pellet was resuspended in the SDS-PAGE loading buffer with no reducing agent. After 30 minutes, the cells were removed and all samples applied on SDS-PAGE gels. Two identical gels were run, one of which was silver-stained, and the proteins

from the other were blotted on a PVDF (polyvinylidene fluoride) membrane. The free binding sites on the membrane were blocked using 3% BSA, and the membrane was incubated either with 0.02 µg/ml of the Anti-Glycoprotein-340 primary antibody (anti-gp340, HYB213-06, Antibodyshop, Gentofte, Denmark) for 60 minutes (III, detection of gp340) or with strepavidin conjugated HRP (0.01 µg/ml) (III, V, detection of LPO). In case of gp340, after the washes the membrane was incubated with the secondary antibody, anti-mouse Ig-conjugated horseradish peroxidase (HRP, Dako, Denmark) for 60 minutes. All the biotinylated proteins were detected with ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Little Chalfont Buckinghamshire, UK) according to the manufacturer's instructions.

Degradation of lactoperoxidase (III)

To test for the degradation of peroxidase, the labelled or unlabelled protein (40 µg/ml) was mixed with 2 x 10^9 CFU/ml of washed, overnight-grown *L. rhamnosus* GG, *L. casei* Shirota or *S. mutans* MT 8148 (for comparison) in a phosphate buffer (pH 6.8) supplemented with 1 mM $CaCl_2$. In control experiments, the bacteria or lactoperoxidase were mixed in the buffer alone. After a 60 or 180-minute incubation at RT, the bacteria were removed by centrifugation (10 000 x g, 5 minutes) and the supernatants mixed with the SDS loading buffer, heated for 5 minutes at 95ºC and applied to SDS-PAGE gels. Two identical gels were run, one of which was silver-stained, and the proteins from the other were blotted on a PVDF (polyvinylidene fluoride) membrane and biotinylated proteins detected as described above.

4.10. Activation of the peroxidase system and its effects (I, IV, V)

Effects of the activated peroxidase system on the acid production by lactobacilli, as well as the effects studied in the saliva are described in Chapters 4.5 and 4.8.

Effects on the growth of the lactobacilli (V)

Overnight-grown lactobacilli were inoculated into MRS supplemented with LPO (5 µg/ml), KSCN (1mM) and glucose (50 mM). The growth was followed for 24 hours at 37ºC by measuring the optical density (OD) at 600 nm first every two hours (0-8 h) and then after 12 and 24 hours of growth. The experiments were repeated in the presence of DTT and catalase.

Effect of the lactoperoxidase system on the viability of H. pylori in Solution I (IV)

H. pylori ATCC 43504 and five clinical isolates (A-E, IV) were used in the assays. For test reactions, 100 µl of bacterial suspension (10^9 CFU/ml) was added to 900 µl of Solution I (pH 5.0 or 6.5), to which the components of LPO system (5 µg LPO/ml, 1 mM KSCN and 10, 100, 300 or 500 µM H_2O_2) were added. After a 1-hour incubation at 37°C in a candle jar, 5 µl of DTT was added. Aliquots of 100 µl were withdrawn before incubation from the control tube and after the incubation from every tube. The bacteria were plated and the colonies counted after three days of incubation. At pH 5.0 the experiments were made only with *H. pylori* ATCC 43504.

Effects of the* Lactobacillus-*activated peroxidase system on* Helicobacter pylori *and* Aggregatibacter actinomycetemcomitans *(V)

The experiments were made in 12-well plates with 1 ml cell culture inserts with a porous base (0.4 µm, Millipore, Billerica, MA, USA). *H. pylori* or *A. actinomycetemcomitans* resuspended in FMM together with the components of the LPO system and glucose (together or separately) were placed into the wells and lactobacilli, together with the same components of the LPO system and glucose, into the insert. The total volumes were 1.2 ml outside and 0.5 ml inside the insert. The final concentrations were 1 mM of KSCN, 5 µg/ml of LPO and 10 mM of glucose. Controls, 1mM KSCN and 5 µg/ml of LPO, (both alone and combined), in FMM at pH 4.0 and pH 7 were included in the test with *A. actinomycetemcomitans*. After one hour of incubation at 37°C at 100 revolutions per minute rotation, 10 µl of DTT (final concentration of 1 mM) was added to prevent any oxidation reactions. The bacterial suspensions were decimally diluted with PBS, and *H. pylori* was enumerated on Brucella and *A. actinomycetemcomitans* on Blood agar. All experiments were also repeated in a buffered medium, Solution I.

4.11. Statistics

All statistical analyses performed are described in detail in I-IV. When parametric tests were appropriate, the Student's t-test or ANOVA (Analysis of Variance) with subsequent pair-wise comparisons were used. The Mann-Whitney U-test was used as a non-parametric test. The Spearman correlation was used to asses the association between the binding to HA and binding to MTW (I). The level of statistical significance was always set at 0.05.

5. RESULTS AND DISCUSSION

5.1. Colonisation potential

To be able to colonise an environment, micro-organisms have to be able to survive in the environment in question and to adhere to a surface in it or grow faster than the dilution rate. In the oral cavity, microbes have to, for example, resist the defence factors of the saliva, and unless they adhere to the oral surfaces they are rapidly swallowed. Bacteria can attach to immobilized salivary proteins, i.e. to the acquired pellicle, to epithelial cells, or (co)adhere with other bacteria already adherent to a surface. The ability to colonise the oral cavity can be considered essential for the bacteria to exert their effects on the oral cavity. Thus, colonisation potential is an important aspect when either the safety or potential probiotic characteristics of a bacterium are considered.

5.1.1. Survival of lactobacilli and bifidobacteria in saliva and in the presence of the peroxidase system (I, V)

The survival of lactobacilli and bifidobacteria in saliva was tested with 20 probiotic, dairy and faecal strains (Table 2, indicated with I). All strains tested survived in the saliva. None of them grew in saliva and only two of the tested strains showed decreased CFU counts after 24 hours of incubation. In addition, the sensitivity of bacteria to the activated peroxidase system in the saliva was tested with two probiotic strains, *L. rhamnosus* GG and *B. lactis* Bb12. Peroxidases in the saliva generate antimicrobial hypothiocyanite ($HOSCN/OSCN^-$) from H_2O_2 and SCN^-. An addition of 50 μM hydrogen peroxide to the saliva resulted in 48 - 50 μM $HOSCN/OSCN^-$, which is equivalent to *in vivo* concentrations detected in human whole saliva (Pruitt *et al.*, 1983). This amount of $HOSCN/OSCN^-$ was not expected to affect the viability of lactobacilli in saliva (Lumikari *et al.*, 1991) and, indeed, the viability of *L. rhamnosus* GG was not affected. The sensitivity of bifidobacteria to the peroxidase system has not been reported previously, but the viability of *B. lactis* Bb12 was also unaffected.

The effects of the components of the peroxidase system on the growth of the probiotic bacteria were studied in MRS (V, Fig. 2). For this purpose, three probiotic *Lactobacillus* strains with different H_2O_2-generating activities were selected. When lactoperoxidase (LPO) and KSCN were added into the growth medium, *L. plantarum* 299v, which produced high amounts of H_2O_2, grew more slowly, but the stationary phase of growth was reached at the same bacterial density as without the addition of the components of the peroxidase system. The growth of *L. reuteri* SD2112, which produced moderate amounts of H_2O_2, was not inhibited by the components of the LPO system. Interestingly, the growth of *L. rhamnosus* GG, which did not produce measurable amounts of H_2O_2, was strongly inhibited, when LPO, together with KSCN, was added into the growth medium. Furthermore, in contrast to *L. plantarum* 299v, the growth reached the stationary phase at a significantly lower bacterial density than in the control. Similar growth inhibition has been described earlier for some strains of lactobacilli and streptococci, but they were strains generating H_2O_2 (Thomas *et al.*, 1981, Slowey *et al.*, 1968). The effects of the

activated peroxidase system on the metabolism of the lactobacilli are discussed in more detail in Chapter 5.2.2, p. 51.

5.1.2. In vitro *binding of lactobacilli and bifidobacteria on oral surfaces*

5.1.2.1. Binding to immobilised salivary proteins (I)

When the persistence of a bacterium in the oral cavity is discussed, binding to oral surfaces is of primary importance. The binding of 20 *Lactobacillus* and *Bifidobacterium* strains on immobilised salivary proteins was studied with two different adhesion assay methods: a MTW assay that is commonly used in studies with bacterial adhesion, and an HA assay which mimics the human enamel. Most of the strains studied behaved similarly in both adhesion assays (Fig. 2, Spearman correlation 0.700, $p < 0.001$). However, a few of the strains studied behaved differently on different surfaces. One reason might be that the different surfaces, the more hydrophobic polystyrene and the hydrophilic HA, attract different bacteria or, more likely, were coated with different salivary proteins. Although there are differences in the pellicle composition *in vivo* and on HA *in vitro* (Carlen *et al.*, 1998, Yao *et al.*, 2001), HA is often used as a model for bacterial adhesion on the tooth surface.

Individual differences and the differences in the ability of parotid and unstimulated whole salivas to mediate bacterial binding were tested with *L. rhamnosus* GG. The adherence of *L. rhamnosus* GG was high to saliva-coated HA, regardless of the saliva used, but with three of the five individuals who supplied the saliva, the binding was stronger to the whole saliva than to parotid saliva-coated HA (I, Fig. 1). The protein composition of these salivas is different and they can mediate oral bacterial adhesion differently (Carlen *et al.*, 1998). Albumin, for example, is regarded as a serum filtrate to the mouth; therefore, the parotid saliva does not normally contain it (Schenkels *et al.*, 1995). Interestingly, we found that an addition of albumin to the parotid saliva diminished the difference between the parotid and whole saliva. In addition, there were clear differences in the adhesion of *L. rhamnosus* GG on HA coated with the salivas of different individuals. There can be large individual variations in the saliva composition, so the differences are not surprising, but it is important to keep this in mind when considering the colonisation potential of bacteria. Individual differences in the oral colonisation of probiotic bacteria are also obvious *in vivo* (Yli-Knuuttila *et al.*, 2006, Caglar *et al.*, 2009, Krasse *et al.*, 2006), although most probably other factors than the saliva composition are behind these differences.

Large variation in the binding of the lactobacilli to saliva-coated surfaces was observed: However, the binding of all the bifidobacteria was low on saliva-coated HA (Fig. 2). The probiotic strains did not stand out as a high (or low) binding group among the strains studied, but all of the dairy strains were in the low-binding group. This is in accordance with a recent publication on yoghurt fermentation starter strains (Stamatova *et al.*, 2009). However, significant differences have been observed in the ability of dairy *Lc. lactis* and *S. thermophilus* strains to bind and influence biofilm formation (Comelli *et al.*, 2002). The good attachment ability of a strain to the intestinal mucus or epithelial cells has been considered an important criterion for probiotic micro-organisms (Ouwehand *et al.*, 2002).

A large variation in the binding of the bacteria to saliva-coated surfaces was observed, though one of the selection criteria of the faecal strains for this study was their good adherence on the intestinal mucus (Apostolou *et al.*, 2001a, He *et al.*, 2001b). Thus, the binding of bacteria to intestinal mucus does not automatically correlate directly with their binding to immobilised salivary proteins. On the other hand, all strains that bound well to both, the saliva-coated HA and MTW, had a tendency to bind also to other surfaces (I, Table 2). Yet, the binding to saliva-coated HA was higher than to BSA-coated HA. This finding, together with the observation that different saliva secretions mediated bacterial binding differently, indicates at least partial specificity of the bacteria to specific salivary proteins.

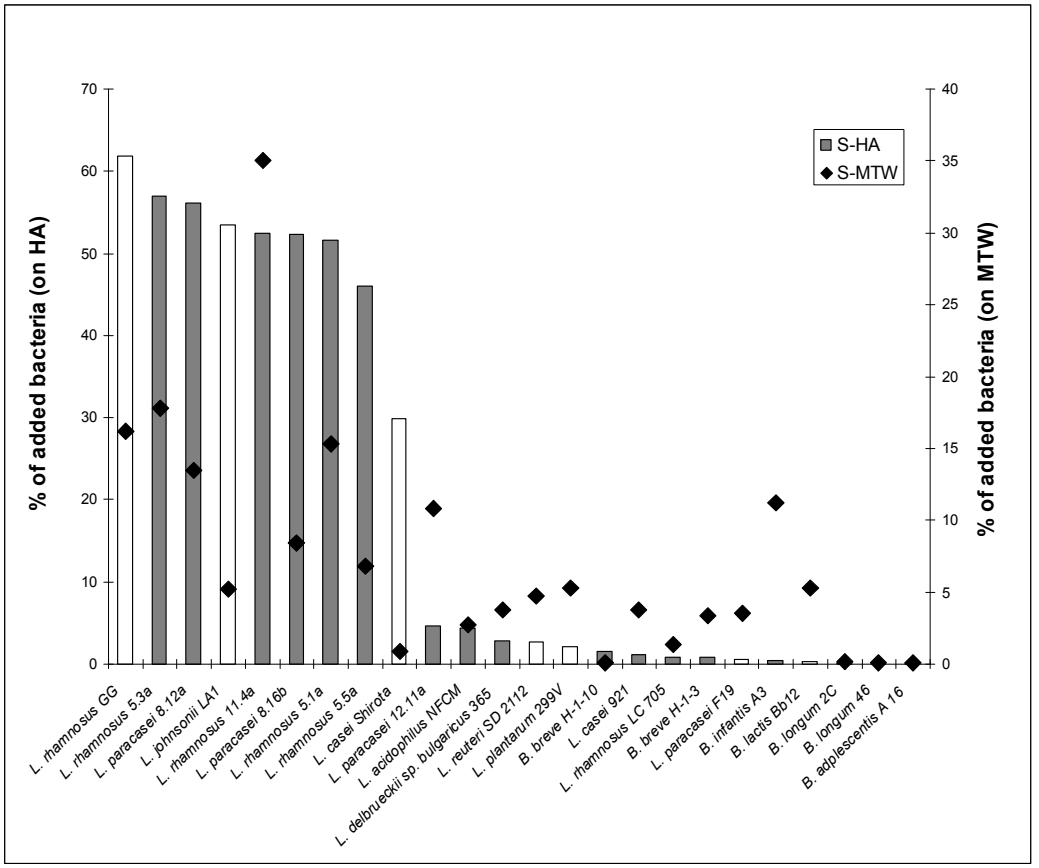

Figure 2. Adhesion of lactobacilli and bifidobacteria on saliva-coated hydroxyapatite (S-HA) and saliva-coated microtitre wells (S-MTW). Probiotic strains are indicated with white bars.

5.1.2.2. Binding to saliva and Fusobacterium nucleatum *or saliva and* Streptococcus mutans *or* gordonii*-coated hydroxyapatite (I, III)*

Interactions between the oral bacteria play a major role in the formation of dental plaque (Filoche *et al.*, 2004, Foster & Kolenbrander, 2004, Palmer *et al.*, 2003). In this respect, *F. nucleatum* is one of the key organisms. Five *Lactobacillus* and two *Bifidobacterium* strains with different binding properties in the two adhesion assays (I) were selected for further experiments for the interactions with *F. nucleatum, S. mutans* and *S. gordonii* (Table 2, indicated with I* or III).

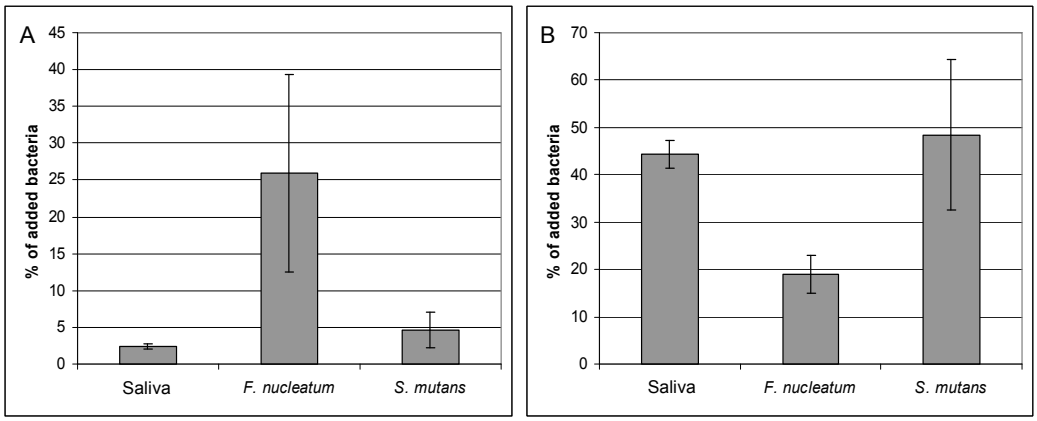

Figure 3. Adhesion of *Bifidobacterium lactis* Bb12 (A) and *Lactobacillus rhamnosus* GG (B) to saliva-coated hydroxyapatite after adhesion of *Fusobacterium nucleatum* or *Streptococcus mutans*

F. nucleatum, S. mutans and *S. gordonii* alone bound well to saliva-coated HA. Streptococci did not affect the adherence of the lactobacilli or *B. lactis* Bb12, but an *F. nucleatum* coating on HA significantly enhanced the binding of the bifidobacteria that did not bind to saliva-coated HA. In contrast, the adherence of high-binding lactobacilli was diminished (Fig. 3). As saliva may influence the interaction between the bacteria (Lamont & Rosan, 1990), the effect of saliva on attached *F. nucleatum* was tested, but no effect was detected. In general, the *in vitro* adherence of the bifidobacteria to host surfaces is poor when compared to lactobacilli, as seen in results presented herein and by others (Apostolou *et al.*, 2001a, He *et al.*, 2001b). They are, however, a part of the normal healthy intestinal microbiota and are also detected in saliva as well as in different dental caries lesions (Aas *et al.*, 2008, Crociani *et al.*, 1996, Mantzourani *et al.*, 2009, Sanyal & Russell, 1978). The increased adherence of bifidobacteria is in accordance with the suggestion that other bacteria influence the adherence of bifidobacteria in the intestine. For example, *L. rhamnosus* GG and *L. delbrueckii* subsp. *bulgaricus* significantly enhanced the adherence of *B. lactis* Bb12 to intestinal mucus *in vitro* (Ouwehand *et al.*, 2000). In addition, consumption of *L. rhamnosus* GG increased the number of bifidobacteria in the faeces of healthy test subjects (Apostolou *et al.*, 2001b). It is also possible that the expression of some genes important for bifidobacterial adherence is down-regulated *in vitro* as environmental conditions are known to influence the expression of genes important for bacterial adhesion (El-Sabaeny *et al.*, 2000).

On the other hand, the decreased adherence of high-binding lactobacilli in the presence of *F. nucleatum* suggests that these strains may compete for the same binding sites on saliva-coated HA. It is also possible that the *F. nucleatum* cells as long, sickle-shaped bacteria simply physically block the binding of these lactobacilli. *F. nucleatum* did not affect the binding of low-binding lactobacilli, but it has recently been shown that a *Lactobacillus* strain not able to form biofilm in a monoculture was successfully established in a saliva-derived biofilm model (Pham *et al.*, 2009). Furthermore, *L. reuteri* ATCC 55730 (=SD2112), which could not adhere in the model used in this study, can be detected in the mouths of people during the time they are using products containing it (Caglar *et al.*,

2009). Thus, it can be assumed that other bacteria, both in the intestine and in the oral cavity, influence the colonisation potential of probiotics in these sites specifically.

5.1.2.3. Binding of lactobacilli and bifidobacteria to buccal epithelial cells (I)

The binding of lactobacilli and bifidobacteria to buccal epithelial cells was studied in the two adhesion assays using five *Lactobacillus* and two *Bifidobacterium* strains with different binding properties. The *L. rhamnosus* strains GG and 5.1a bound well to buccal epithelial cells. The bindings of the other tested lactobacilli or bifidobacteria were significantly lower, although a few bacteria were seen attached to the buccal epithelial cells. Of the studied probiotic strains, *L. rhamnosus* GG was the only strain which attached to buccal epithelial cells. Interestingly, this strain was used in a probiotic product which decreased the oral *Candida* in the elderly, although no changes in mucosal lesions were seen (Hatakka *et al.*, 2007).

5.1.3. Conclusions

All *Lactobacillus* and *Bifidobacterium* strains survived well in saliva, but there were significant differences in their ability to bind to the oral surfaces. The probiotic strains did not stand out as high or low-binders, and a similar diversity was observed in the group of faecal isolates. None of the dairy strains adhered to saliva-coated surfaces. Good adhesion to the intestinal mucus did not correlate with good adherence to the simulated oral surfaces. In addition, bifidobacteria which did not bind to saliva-coated surfaces bound well to *F. nucleatum*-coated HA, implicating other oral bacteria in modulating their colonisation potential. Furthermore, although the activated peroxidase system was not bactericidal against lactobacilli or bifidobacteria, bacteriostatic effects were observed, with clear differences in sensitivity between the strains. Thus, despite their ability to bind to oral surfaces, the growth of some strains might be inhibited in the oral environment. In this study, the most potent coloniser of the oral cavity as evaluated by survival and bacterial binding was *L. rhamnosus* GG. *In vivo*, it can be detected in the oral cavity of most people consuming products containing it (Yli-Knuuttila *et al.*, 2006, Meurman *et al.*, 1994).

During the last few years, the oral colonisation of probiotic bacteria has been studied *in vivo*. In accordance with the results presented here, differences between different strains and individuals are obvious in these studies (Yli-Knuuttila *et al.*, 2006, Caglar *et al.*, 2009, Petti *et al.*, 2001, Krasse *et al.*, 2006, Busscher *et al.*, 1999). Furthermore, as the oral clearance is dependent on food texture, it can be hypothesised that oral colonisation of probiotic bacteria from different products is also different. Indeed, some of the observed differences in oral colonisation of the same bacterial strain could perhaps be explained by use of different products (Yli-Knuuttila *et al.*, 2006, Meurman *et al.*, 1994). The oral colonisation by probiotic bacteria *in vivo* has been studied using saliva samples, but in this study, a HA model mimicking colonisation on the tooth surface was used. It can not be excluded that some of the discrepancies between the *in vivo* observations and results of this study stem from the distribution of probiotic bacteria in the oral cavity. After all, the microbiota of unstimulated whole saliva is more similar to that of the tongue than the dental plaque (Mager *et al.* 2003). There is no information available regarding oral

colonisation by probiotic strains in children, although probiotic bacteria are administered even to newborns (Taipale *et al.*, 2007, Rautava, 2007) and the developing microbiota is more likely to be affected by the probiotic bacteria for a longer time. Indeed, with *L. rhamnosus* GG it has been shown that although in most cases the consumed probiotic bacteria remain in the oral cavity only for the time the product containing the bacteria is used, a long-term colonisation is also occasionally possible though uncommon (Yli-Knuuttila *et al.*, 2006).

5.2. Acidogenicity

Lactobacillus and *Bifidobacterium*, the bacterial genera most often used in probiotic products, are acidogenic, and, particularly in the case of lactobacilli, also aciduric. Indeed, generation of organic acids is one of the desirable attributes of the probiotics in the intestine (Servin, 2004). On the other hand, the acid production from fermentable carbohydrates is one of the most important cariogenic traits. Furthermore, acidogenic and aciduric species can inhibit other competing organisms and make their local environment, for example, the dental plaque, even more acidic. Yet, some species of *Lactobacillus* and *Bifidobacterium* may also have a role in maintaining health by promoting a microbiological balance in the oral cavity (Simark-Mattsson *et al.*, 2007, Hojo *et al.*, 2007a, Kõll-Klais *et al.*, 2005a), and oral defence factors, such as the peroxidase system may inhibit the acidogenicity of bacteria. Thus, before recommending probiotic bacteria for oral health care, their safety, i.e. their inability to cause diseases, must be ascertained.

5.2.1. Acid production by lactobacilli and bifidobacteria from sugars and sugar alcohols (II)

Acid production of 14 probiotic and dairy *Lactobacillus* and *Bifidobacterium* strains (Table 2, indicated with II) in Fermentation Minimal Medium (FMM) was followed for 30 minutes. All of the strains produced acids from glucose, and the decrease in pH was comparable to that produced by *S. mutans*. With all the strains, the pH sank below 5 in five minutes, and the final pH after 30 minutes of incubation was below 4 (Figure 4, see also II, Table 1). Thus, all strains tested were acidogenic. Although glucose levels are relatively low in saliva, within the dental plaque, glucose can be available for the bacteria. It is formed by sucrases of the bacteria in whole saliva and in oral biofilms (Fiehn *et al.*, 1986). With sugars, other than glucose, or with sugar alcohols, the reduction of pH was slower. Apart from *L. rhamnosus* GG, all of the *Lactobacillus* strains, but only one of the four *Bifidobacterium* strains tested (*B. lactis* Bb12), caused a significant decrease in pH with lactose. Sucrose was used by six of the ten *Lactobacillus* strains and only by *B. lactis* Bb12 of the bifidobacteria. The results obtained in this study lead to somewhat different conclusions than reported recently by Hedberg *et al.* (2008). They concluded that the probiotic lactobacilli have a generally weak metabolic activity with dietary sugars. In general, sugars are considered to be characteristic substrates for the metabolism of lactic acid bacteria (Zaunmuller *et al.*, 2006). The differences between the results of this study and of Hedberg *et al.* (2008) can be explained by the different methodologies used. In this study, the pH of an unbuffered medium was monitored for 30 minutes after the exposure of starved bacteria to sugar or sugar alcohol; Hedberg *et al.* (2008) used a fermentation assay

(24 h, 48 h, 72 h) in a phosphate-buffered medium also containing proteins and peptides. Neither of the assays closely resemble the conditions in the oral cavity. The assay used in this study may lead to too-pessimistic conclusions in respect to caries as the assay mixture contained no buffering, which is not the situation in the oral cavity. However, an assay in which not even glucose is fermented by a *Lactobacillus* strain (Hedberg *et al.*, 2008) may be unsuitable for ruling out deleterious effects on the teeth.

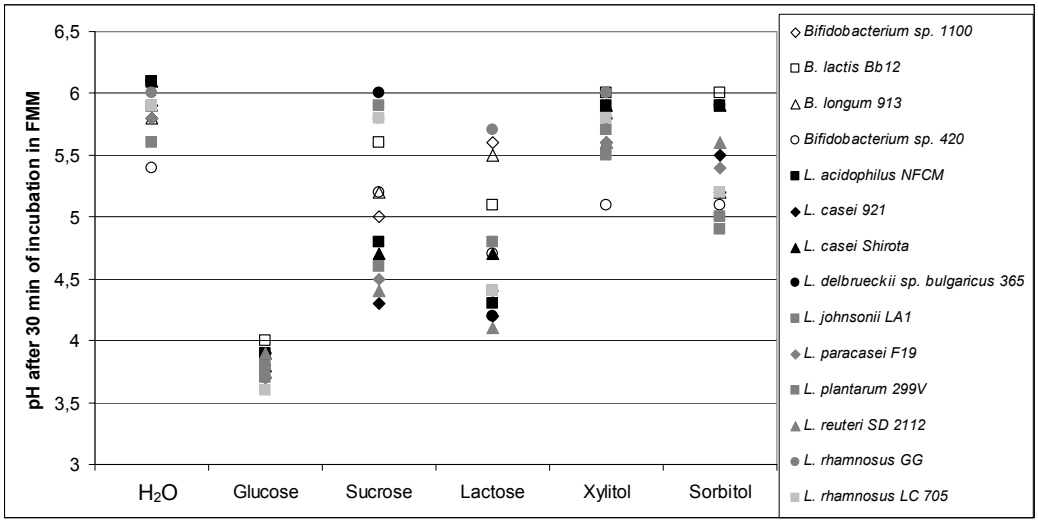

Figure 4. The final pH of Fermentation Minimal Medium after 30 minutes of incubation with lactobacilli and bifidobacteria together with different sugars or sugar alcohols (See also II).

A statistically significant decrease in pH was also observed with some strains with xylitol or sorbitol (II, Table 1). The changes in pH with xylitol and sorbitol were generally minor; therefore, most probably, they do not have any clinical significance. Some *Lactobacillus* strains have also been previously shown to be able to produce acids from sorbitol and xylitol (Badet *et al.*, 2001, Klewicki & Klewicka, 2004). In addition, some oral lactobacilli have been shown to adapt to xylitol during long term *in vitro* exposure (Badet *et al.*, 2004), although no adaptation has been observed *in vivo* (Mäkinen *et al.*, 1985).

5.2.2. Effects of the lactoperoxidase system on acid production (V)

$HOSCN/OSCN^-$ generated by the peroxidase system inhibits the glucose metabolism of different lactic acid bacteria (Mansson-Rahemtulla *et al.*, 1987, Carlsson *et al.*, 1983). The effects of the LPO system on the acid production by the probiotic bacteria were therefore tested with three probiotic *Lactobacillus* strains which were different producers of H_2O_2: *L. plantarum* 299v, which was the most efficient producer of H_2O_2, *L. reuteri* SD2112, which produced small amounts of H_2O_2, and *L. rhamnosus* GG, which did not produce measurable amounts of H_2O_2 in the initial experiments.

The acid production was monitored again in FMM for 30 minutes, but this time, the components of the LPO system, together or separately, were also added to the reaction medium. The complete LPO system inhibited the acid production of all three probiotic

strains tested, but there were marked differences in the sensitivity between the strains. *L. reuteri* SD2112 was inhibited only at a lower bacterial concentration (10^8 CFU/ml), but at a higher concentration (10^9 CFU/ml) it overcame the effect of the complete LPO system, and acids were produced efficiently. With the strains *L. rhamnosus* GG and *L. plantarum* 299v, the amount of bacteria used did not affect the efficacy of the inhibition. Furthermore, the acid production of *L. rhamnosus* GG and *L. plantarum* 299v, but not that of *L. reuteri* SD2112, was also inhibited when only the LPO enzyme and KSCN, but no H_2O_2, were added. When a higher amount (10^9 CFU/ml) of bacteria was used, LPO alone also inhibited the acid production of *L. plantarum* 299v. As this strain was the most efficient producer of H_2O_2 in these experiments, this suggests that with high concentrations of H_2O_2 other substrates than KSCN were also used in the LPO-catalysed reaction. The complete LPO system inhibited the acid production of *S. mutans* MT 8148 used as a control regardless of the amount of bacteria used, but KSCN and LPO without any added H_2O_2 had no effect. Thus, the responses towards the self-induced activation of the LPO system were not in accordance with the amount of H_2O_2 released by the bacterial strain.

Addition of catalase diminished the effect of the complete LPO system (LPO, KSCN and H_2O_2) but had hardly any effect on LPO together with KSCN. This suggests that H_2O_2 produced by the bacteria reacted before interacting with the catalase in the medium. Indeed, LPO was bound by the lactobacilli in FMM (V, see also p. 55). Thus, probably the entire LPO-catalysed reaction had already taken place on the bacterial surface. Both LPO and SPO bind readily to different surfaces in an active form (Pruitt *et al.*, 1979). Addition of DTT totally eliminated the effects of LPO and KSCN or the complete LPO system. DTT reduces HOSCN/$OSCN^-$, the antimicrobial component of the used peroxidase system and the disulfide bridges of proteins, thus most probably affecting the activity of the LPO enzyme.

It was hypothesised that the strains with efficient H_2O_2 production would activate the peroxidase system and thereby their metabolism would be affected. Surprisingly, *L. rhamnosus* GG was sensitive to LPO and KSCN. The growth and acid production of *L. rhamnosus* GG were inhibited, although no H_2O_2 production could be measured. This suggests that the LPO system was activated by something other than H_2O_2. The killing of *F. nucleatum* by myeloperoxidase and I^- or Cl^- with no added H_2O_2 has been reported (Ihalin *et al.*, 2001); in that study, the authors suggested the presence of another oxidative substrate, but it was not analysed further. In addition, oxidation reactions are possibly also catalysed by LPO in the absence of H_2O_2 (Bonini *et al.*, 2007). On the other hand, it is possible that in contrast to previous reports (Pridmore *et al.*, 2008) and the results presented herein, *L. rhamnosus* GG produces H_2O_2. H_2O_2 could have been degraded faster than the detection rate or possibly the amount produced was below the detection limit in the assays used. It can be hypothesised that minor amounts of H_2O_2 could be sufficient to activate the LPO system as the cell-bound peroxidase enzyme can utilize the substrate before it is diluted into the medium. Likewise, HOSCN/$OSCN^-$, the product of the enzymatic reaction, is formed near the bacterial surface. Indeed, the LPO system activated by *L. rhamnosus* GG inhibited only its own metabolism, but LPO did not enhance the antibacterial activity of *L. rhamnosus* GG against other bacteria (V, see also p. 59–60). Furthermore, streptococcal strains producing no or only small amounts of H_2O_2 are more sensitive to the $OSCN^-$ than strains with abundant H_2O_2 production due

to the lack of sufficient activity of NADH-$OSCN^-$ oxidoreductase (Carlsson *et al.*, 1983). In accordance with this, *L. rhamnosus* GG can be very sensitive towards $OSCN^-$, so only minor amounts are needed for its inhibition.

5.2.3. Conclusions

Lactobacilli and bifidobacteria are acidogenic; as a consequence, the effects of probiotic bacteria of those genera on dental health need to be evaluated. According to Marsh (2003), dental diseases, such as caries, need not have a specific microbial aetiology, but any species with the relevant traits can contribute to the disease process. Indeed, acidogenic lactobacilli and bifidobacteria are strongly associated with the caries process (Beighton, 2005). However, acid production alone does not make a bacterium cariogenic, but other factors, such as the ability to bind to tooth surfaces or the dental plaque are required as well (Tanzer *et al.*, 2001). Similarly, the host factors need to be taken into account. For example, if *L. rhamnosus* GG is as sensitive to the peroxidase system *in vivo* as in the *in vitro* experiments presented in this study, the peroxidase system could protect the enamel from the potentially harmful effects of this probiotic bacterium. It can be speculated that by adhering (I, III) but being metabolically passive, *L. rhamnosus* GG could physically block the binding of other strains, such as cariogenic *S. mutans* (III), and thereby influence their colonisation. Indeed, *L. rhamnosus* GG may reduce the caries risk when used at the right age (Näse *et al.*, 2001).

5.3. Saliva-mediated effects of probiotic bacteria on other bacteria (III, V)

Understanding how probiotic bacteria influence the oral microbial ecology is important if probiotic bacteria are proposed for oral health purposes. The possible ways for probiotic bacteria to affect the oral ecology could be similar to those described in the urogenital tract and the intestine, such as displacement of pathogens (Vesterlund *et al.*, 2006), competition for binding sites (Neeser *et al.*, 2000, Pretzer *et al.*, 2005), nutrients and growth factors (Hojo *et al.*, 2007b), secretion of antibacterial molecules (Silva *et al.*, 1987, Morita *et al.*, 2008) or activation of the peroxidase system (Klebanoff *et al.*, 1991). In addition, in the oral cavity, the tooth surfaces are rapidly coated with a salivary film, the pellicle, which provides binding receptors for a number of bacteria. Modifications induced by probiotic bacteria in the composition of this pellicle may alter its microbial binding preferences. Acidogenicity has already been discussed in the previous section.

5.3.1. Effects on the salivary pellicle and interaction with salivary agglutinin gp340 and peroxidase

5.3.1.1. Effect of the Lactobacillus, Bifidobacterium *and* Lactococcus *strains on the pellicle composition (III)*

The effects of probiotic bacteria on the salivary pellicle composition were studied with probiotic strains *L. rhamnosus* GG, *L. casei* Shirota, *L. reuteri* SD2112 and *B. lactis* Bb12 (Table 2, indicated with III). In addition, *L. casei* ATCC 11578 and *Lc. lactis* MG 1363 were used as controls. All strains studied had an apparent effect on the protein composition

of the salivary pellicle on HA as demonstrated by SDS-PAGE (III, Fig. 1) and by using the adherence of streptococci as a biological indicator (p. 57). In addition, after the saliva had been incubated with high amounts of bacteria, particularly with *L. reuterii* SD2112 and *B. lactis* Bb12, new protein bands were visible in the gels. New bands were also seen in the silver-stained gels with control samples, which were taken from supernatants of a bacterial suspension in which the bacteria were incubated in a buffer.

The amounts of salivary agglutinin gp340 and SPO were clearly diminished or even totally lost in pellicles made of the saliva incubated first with the probiotic bacteria or *L. casei* ATCC 11578. A clearly weaker effect on the amount of salivary agglutinin was observed with *Lc. lactis* MG 1363, but this control strain, too, removed SPO from the pellicle. Changes in the pellicle protein composition may affect the composition of the developing dental plaque. Adhesion of the primary colonisers is determined by the presence of suitable receptors and adhesins in the pellicle (Xu *et al.*, 2007, Clark *et al.*, 1978). Thus, the removal of salivary agglutinin gp340, an important adhesion receptor in the pellicle (Carlen & Olsson, 1995), may modify the microbial binding preferences of the pellicle. Primary colonisers of the dental plaque are important in modifying the environment and influencing the binding of the subsequent bacteria. SPO, on the other hand, does not facilitate bacterial binding, but may affect *S. mutans* adherence by blocking its binding or inhibiting its glucosyltransferase activity (Roger *et al.*, 1994, Korpela *et al.*, 2002). Finally, as a part of a defence system, it can influence the functions, including acidogenicity, of the dental plaque.

5.3.1.2. Interactions of probiotic bacteria with the salivary agglutinin (III)

The aggregation of lactobacilli and bifidobacteria was studied in parotid saliva using the strains *L. rhamnosus* GG, *L. casei* Shirota, *L. reuteri* SD2112 and *B. lactis* Bb12. *L. casei* ATCC 11578 and *Lc. lactis* MG 1363 were used as controls (Table 2, indicated with III). All bacteria, except *L. reuteri* SD2112, formed visible aggregates in the presence of parotid saliva, although only *L. rhamnosus* GG and *L. casei* Shirota adhered to parotid saliva-coated surfaces. The protein composition of pure parotid gland secretion is different from the protein composition of the pellicle (Al-Hashimi & Levine, 1989). In addition, some microbes may have different binding affinities towards the fluid and surface-bound salivary proteins (O'Sullivan *et al.*, 2000, Loimaranta *et al.*, 2005).

All bacterial strains tested bound purified salivary agglutinin gp340, and the bound protein could be released from the bacteria. The binding of salivary agglutinin took place in less than 10 minutes with the strains *L. rhamnosus* GG, *L. casei* Shirota, *B. lactis* Bb12 and *L. casei* ATCC 11578, and also the amount of salivary agglutinin released from the bacteria was approximately the same regardless of the incubation time. *L. reuterii* SD2112 and *Lc. lactis* MG 1363 bound the salivary agglutinin somewhat more slowly, the binding occurring in approximately 30 minutes. The salivary agglutinin mediates the aggregation and adhesion of several bacterial species, including different *Streptococcus* and *Actinomyces* species and *Helicobacter pylori* (Prakobphol *et al.*, 2000, Loimaranta *et al.*, 2005). In the liquid phase, the binding of salivary agglutinin gp340 has been associated with the aggregation and possibly enhanced clearance of bacteria from the oral cavity, although calcium-dependent binding of bacteria to a parotid saliva agglutinin

(possibly gp340) without aggregating has been reported (Rundegren, 1986). Interestingly, all the strains tested here bound salivary agglutinin gp340, regardless of their adhesion or aggregation properties.

5.3.1.3. Binding and degradation of the salivary peroxidase (III, V)

The amount of salivary peroxidase was also reduced in those pellicles formed of parotid saliva incubated with the probiotic bacteria or the control organisms. The ability of the bacteria to bind peroxidase was tested with purified bovine LPO and with the strains *L. reuteri* SD2112, *L. rhamnosus* GG and *L. plantarum* 299v in the FMM and MRS media. There were no differences in the binding of LPO to the surfaces of the bacteria. All strains bound LPO in FMM, and the enzyme could be released from the bacterial surface. In contrast, virtually no LPO was found on the bacterial surface after incubation of the bacteria in the MRS medium, and accordingly, virtually all of the enzyme was found in MRS. Both SPO and LPO have a tendency to bind to bacterial and other surfaces (Pruitt *et al.*, 1979, Tenovuo *et al.*, 1977). It can be hypothesised that in FMM, where no other proteins were added, LPO would bind more readily. On the other hand, the amount of SPO was clearly decreased in the salivary pellicles made of saliva first incubated with the lactobacilli. Thus, in the human saliva, competition such as that observed in MRS, did not suppress the binding.

The ability of *L. rhamnosus* GG and *L. casei* Shirota to degrade peroxidase was tested with purified bovine lactoperoxidase. After a 60 or 180-minute incubation of LPO with the probiotic lactobacilli, most of the peroxidase was still present in the supernatant. After 180 minutes of incubation with the probiotic bacteria, a novel protein band of approximately 40 kDa originating from LPO (as tested with biotinylated LPO) was visible in silver-stained gels (III, Fig. 3). When the lactoperoxidase was incubated with *S. mutans* MT 8148 or a buffer alone, no such novel protein bands were detected. Although the degradation of lactoperoxidase by oral streptococci has been speculated (Pruitt *et al.*, 1979), degradation of a protein involved in the innate immune system by a probiotic strain was a surprising finding and has not been reported earlier. On the other hand, the proteolytic activity of *L. rhamnosus* GG (Sutas *et al.*, 1996) and also of other lactic acid bacteria (Pihlanto & Korhonen, 2003) is well-known and has been used, for example, to generate bioactive peptides from milk proteins. Similarly, in saliva, antimicrobial peptides can be released by cleavage from proteins that in their intact form may have only limited antimicrobial activity (Bellamy *et al.*, 1992). Although the degradation of the lactoperoxidase was slow in the experimental conditions, the finding cannot be disregarded, and the effects and the exact nature of this proteolytic activity remain to be solved.

5.3.2. Effects on streptococcal adhesion (III)

To study the effects of probiotic bacteria on streptococcal adhesion, probiotic strains with different affinities to saliva-coated surfaces (I) were selected (indicated with III in Table 2) and again *L. casei* ATCC 11578 with no known probiotic properties and *Lc. lactis* MG 1363 were used as controls. The salivary pellicle on HA and the adhesion of two oral streptococci, *S. mutans* strains MT 8148 and Ingbritt and *S. gordonii* DL 1 were used as a model.

5.3.2.1. Effects of the adhered probiotic bacteria on streptococcal adhesion

There were no differences between the *S. mutans* strains MT 8148 and Ingbritt in the adhesion experiments. Probiotic strains which adhered to parotid saliva-coated HA (*L. rhamnosus* GG and *L. casei* Shirota) significantly diminished the adherence of the *S. mutans* strains MT 8148 and Ingbritt when they adhered to HA before the streptococci (Fig. 5). The adherence of *S. gordonii* DL1was reduced only by *L. rhamnosus* GG. Furthermore, the inhibitory effect on *S. gordonii* DL1 was clearly weaker than on *S. mutans* (Fig. 5). Thus, the inhibition of *S. mutans* adhesion was apparently not completely due to non-specific steric hindrance of binding. Although it is possible that the detected difference between the adherences of *S. mutans* and *S. gordonii* was partly due to the better adherence of *S. gordonii*, it seems likely that the lactobacilli occupy specific salivary receptors for *S. mutans* while the receptors for *S. gordonii* remain unoccupied. In fact, *S. gordonii* appears to have a wider pattern of receptors on salivary pellicles than *S. mutans* (Rosan & Lamont, 2000, Ahn *et al.*, 2003). The adhesion of streptococci was inhibited only when the probiotic bacteria were pre-added. When the probiotic strains, or the control strains, and the streptococci were allowed to adhere simultaneously, or when the streptococci were allowed to adhere before the probiotics or the control strains, only *L. casei* ATCC 11578 affected the adherence of the streptococcal strains studied: it slightly inhibited the adherence of *S. mutans*, and it could even release the already bound *S. mutans* and *S. gordonii* cells from the HA. Thus, the competition or exclusion of binding was more efficient than displacement. This is in accordance with the results obtained in a model mimicking the intestine (Lee *et al.*, 2003).

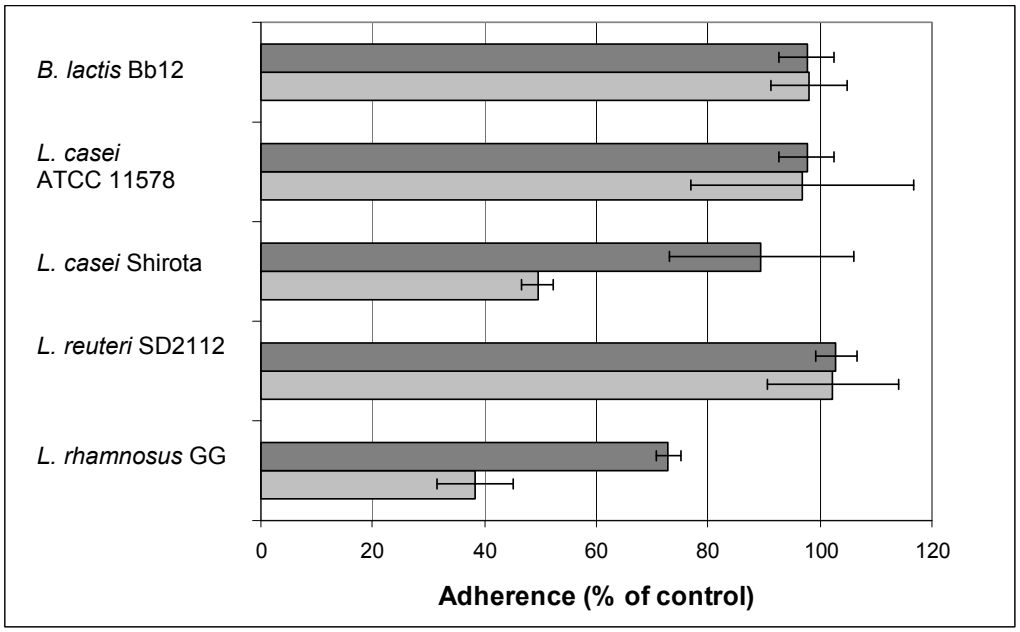

Figure 5. Adhesion of *Streptococcus mutans* MT 8148 (light bars) and *Streptococcus gordonii* DL1 (dark bars) on saliva and *Lactobacillus* or *Bifidobacterium*-coated hydroxyapatite. Adherence to parotid saliva-coated hydroxyapatite functions as a control. Error bars indicate the standard deviation.

5.3.2.2. Effect of alterations in the salivary pellicle on streptococcal adhesion

Incubation of the parotid saliva with *Lactobacillus* strains GG, SD2112, Shirota, ATCC 11578 or *B. lactis* Bb12 before the pellicle was formed significantly decreased the adherence of *S. mutans* strains MT 8148 and Ingbritt, but the adherence of *S. gordonii* DL1 remained unaffected (Fig. 6, see also III, Table 2). These results correlate well with the observed binding of salivary agglutinin gp340, the main receptor for *S. mutans* adhesion on the salivary pellicle, by the probiotic strains. This inhibitory effect could be seen after a one-hour incubation, but it was more apparent after three hours of incubation, indicating that other modifications besides binding of salivary agglutinin gp340 also occurred in the pellicles. Inhibition of *S. mutans* strains MT 8148 and Ingbritt adhesion was also dose-dependent: 10^7 CFU/ml of *Lactobacillus* or *Bifdobacterium* cultures did not have an effect on the adherence of *S. mutans*, but a 10 times higher concentration clearly inhibited the adherence of *S. mutans*. With 2 x 10^9 CFU/ml of *Lactobacillus* or *Bifidobacterium* cultures, the adherence of *S. mutans* was almost eliminated. *Lc. lactis* MG 1363, used as a control, showed a clearly weaker effect on the adherence of *S. mutans*, although with high numbers of bacteria and a long incubation time it also seemed to have some effect on the adherence of *S. mutans*. Control pellicles for these adherence assays were made by first coating HA with parotid saliva and then with a supernatant of a bacterial suspension in a buffer. There was no difference in the adherence of *S. mutans* or *S. gordonii* on the control pellicles.

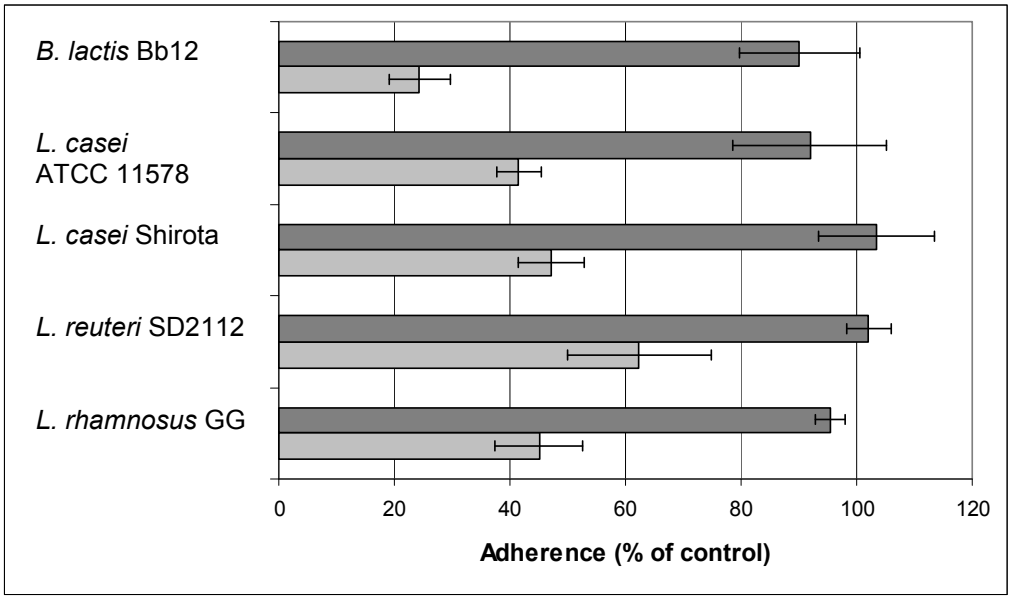

Figure 6 Adhesion of *Streptococcus mutans* MT 8148 (light bars) and *Streptococcus gordonii* DL1 (dark bars) on hydroxyapatite coated with parotid saliva, which was first incubated with lactobacilli or bifidobateria. Their adherence to parotid saliva-coated hydroxyapatite was used as a control. Error bars indicate the standard deviation.

5.3.3. Activation of the peroxidase system (IV, V)

The peroxidase enzymes in the saliva catalyse a reaction between a halide or a pseudohalide and H_2O_2. The antimicrobial HOSCN/$OSCN^-$ formed in the reaction is not only secreted into the oral cavity from the parotid ducts (Geiszt *et al.*, 2003, Pruitt *et al.*, 1983), but peroxidogenic bacteria are also suggested to activate the peroxidase system. To estimate the capability of the peroxidase system activated by probiotic bacteria to affect the viability of other bacteria, two Gram-negative bacteria were used as indicators: *H. pylori*, a bacterium strongly associated with duodenal and gastric ulcers and a risk factor for gastric cancer, and *A. actinomycetemcomitans*, one of the main pathogens in aggressive periodontitis. Their transmission via the saliva is suggested (for review, see Asikainen *et al.* 1997 and Dowsett & Kowolik 2003). In addition, their sensitivity to the LPO system has been studied before (Shin *et al.*, 2002, Ihalin *et al.*, 1998).

5.3.3.1. Sensitivity of Helicobacter pylori to the peroxidase system in buffer and in human saliva (IV)

Before studying the effects of probiotic bacteria on *H. pylori*, its sensitivity to the LPO system was investigated in buffered solution I and in human whole saliva. The *H. pylori*-type strain (ATCC 43504) was used together with five clinical isolates. In solution I, all strains were sensitive to the LPO system, and the effects on the viability were dose-dependent with respect to H_2O_2. The inhibition of the clinical isolates did not differ from each other or that of *H. pylori* ATCC 43504 (IV, Fig. 1). These results are in accordance with those reported by Shin *et al.* (2002). The LPO system inhibited the viability of *H. pylori* significantly at pH 6.5 at H_2O_2 concentrations of 100 μM and above. The inhibition was enhanced at pH 5.0, which is in accordance with the hypothesis that the antimicrobial effects of the LPO system are enhanced in an acidic pH because the protonated, and thus uncharged, HOSCN can diffuse into the bacterial cells (Mansson-Rahemtulla *et al.*, 1987, Lumikari *et al.*, 1991, Thomas *et al.*, 1983). Antimicrobial $OSCN^-$ is in equilibrium with HOSCN, and the reaction has a pK_a of 5.3 (Thomas, 1981). The concentrations of H_2O_2 required to inhibit the viability of *H. pylori* by the LPO system were relatively high when compared to the previous studies with oral Gram-negative anaerobic or capnophilic oral bacteria (Ihalin *et al.*, 2001, Ihalin *et al.*, 1998).

Pooled human saliva was used in the experiments, as individual differences between salivas were significant in respect to their SCN^- and, accordingly, to HOSCN/$OSCN^-$ concentrations (IV, Fig. 2). In contrast to Solution I, in saliva the amount of $OSCN^-$/HOSCN was not linearly dependent on the amount of added H_2O_2, but there was a limit after which the amount of HOSCN/$OSCN^-$ did not increase but decreased with the addition of H_2O_2. Accordingly, the bactericidal effect decreased as the concentration of added H_2O_2 was above that limit (Fig. 7). The inhibition of *H. pylori* by HOSCN/$OSCN^-$ was diminished in the saliva when compared to the buffer. When 100 μM of H_2O_2 was added to the saliva to activate the peroxidase system, the numbers of *H. pylori* were lower when compared to the saliva control after one hour of incubation. The effect of HOSCN/$OSCN^-$ was again greater at pH 5.0 than at pH 6.5. As the lysozyme activity of the saliva was lost during the handling of saliva, the viability of *H. pylori* ATCC 43504 was tested both in the saliva and in saliva with an added physiological concentration of the active lysozyme (IV, Fig. 3). The addition of lysozyme had no, or only a minor,

influence on the viability of *H. pylori,* nor did it enhance the effect of HOSCN/$OSCN^-$, although it has been reported to have a modest inhibitory effect on the growth of *H. pylori* (Dial *et al.*, 1998) and to enhance the effects of the peroxidase system (Lenander-Lumikari *et al.*, 1992).

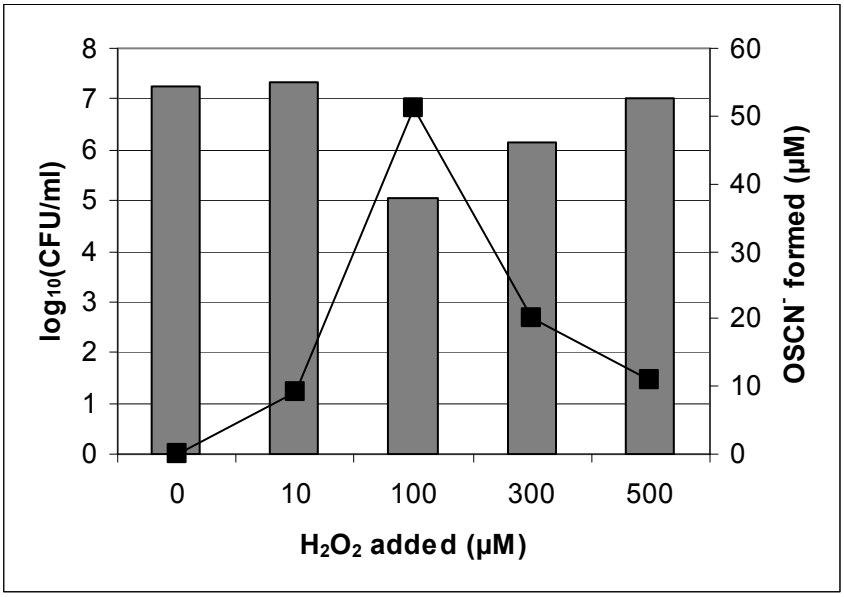

Figure 7. Generation of HOSCN /$OSCN^-$ (-■-) and the viability of *Helicobacter pylori* after an incubation in saliva supplemented with different amounts of H_2O_2

5.3.3.2. Activation of the peroxidase system by probiotic lactobacilli

Probiotic lactobacilli with different activities of H_2O_2 production were used for studying the effects of the Lactobacillus-activated peroxidase system on other bacteria. In the initial testing, *L. plantarum* 299v was the most efficient producer of H_2O_2, *L. reuteri* SD2112 produced smaller amounts of H_2O_2, and with *L. rhamnosus* GG no generation of H_2O_2 was detected.

Only *L. plantarum* 299v could activate the peroxidase system in a manner in which the antimicrobial activity was enhanced when compared to the control reactions. No viable *A. actinomycetemcomitans* were detected after one hour of incubation in FMM together with *L. plantarum* 299v, glucose, LPO and KSCN (V, Table 2). Interestingly, a significant decrease in the CFU levels was found in the control reactions: *A. actinomycetemcomitans* or *H. pylori* were not inhibited by the probiotic lactobacilli alone, but an addition of glucose resulted in a clear inhibition of *A. actinomycetemcomitans* by *L. rhamnosus* GG (V, Table 2). The inhibition was not related to the low pH alone as the inhibition was specific for *L. rhamnosus* GG. Most probably, H_2O_2 was not responsible for this inhibition, although inhibition of pathogens by the H_2O_2-producing *Lactobacillus* strains has been described (Pridmore *et al.*, 2008). In this study, *L. rhamnosus* GG, which does not produce significant amounts of H_2O_2, was the most potent inhibitor. In addition, as catalase-positive organisms, the *A. actinomycetemcomitans* and *H. pylori* strains were

not especially sensitive to H_2O_2 (Miyasaki *et al.*, 1984, Miyasaki *et al.*, 1985). Several lactobacilli are able to inhibit the growth of *A. actinomycetemcomitans* or *H. pylori in vitro* in a species-specific manner (Köll *et al.*, 2008, Lopez-Brea *et al.*, 2008, Rokka *et al.*, 2006, Sgouras *et al.*, 2004). Furthermore, *L. rhamnosus* GG secretes antimicrobial peptides active against a variety of bacteria, including both Gram-negative and Gram-positive species, at a low pH (Silva *et al.*, 1987, Lu *et al.*, 2009). However, the nature of the inhibition seen in this study needs further characterisation.

All of the probiotic bacteria tested, *L. plantarum* 299v, *L. reuteri* SD2112 and *L. rhamnosus* GG, inhibited the viability of *H. pylori* and *A. actinomycetemcomitans* in the presence of glucose and KSCN (V, Table 2). The inhibition of *A. actinomycetemcomitans* by *L. rhmamnosus* GG and *L. plantarum* 299v was extensive. Again, the inhibition was not related only to SCN^- at low pH, as KSCN in FMM at pH 4 resulted in no inhibition, and the inhibition was significantly stronger with *L. rhamnosus* GG and *L. plantarum* 299v than with *L. reuteri* SD2112. The need for glucose suggests the role of one or more metabolic endproducts in the inhibition. In a similar experimental setup, with a buffered medium, in which the LPO-KSCN system was activated by a H_2O_2-producing *S. mitis* strain, no killing of *E. coli, Staphylococcus aureus* or *Candida tropicalis* could be detected with or without the LPO enzyme (Hamon & Klebanoff, 1973). Thus, as no inhibition could be detected in the buffered medium in this study either, acidity appears to be important. On the other hand, the growth inhibition of *C. albicans* by *L. acidophilus* was enhanced by SCN^- (Jack *et al.*, 1990), and the presence of another, not peroxidase but SCN^--dependent anti-microbial system in the parotid saliva is suggested (Dogon & Amdur, 1970). These results concerning *H. pylori* and particularly *A. actinomycetemcomitans* require further experiments to determine the mechanisms behind the bactericidal effects described.

5.3.4. Conclusions

Different probiotic strains, if active in the mouth, would be expected to have different mechanisms of action in the oral cavity as they have different modes of action in other parts of the human body, and some of the observed effects of the probiotic bacteria were indeed strain specific. On the other hand, all of the positive effects of the probiotic bacteria observed in this study were not specific for the probiotic strains but properties common to several species or even genera. Thus, as suggested for probiotics for other purposes (Gueimonde & Salminen, 2006), the probiotic properties of bacteria also need to be determined for each strain separately (or in combinations used in products) for the health benefits in the oral cavity. In addition, previously reported probiotic properties beneficial to gut function do not necessarily correlate with probiotic activity in the oral cavity. Results shown here indicate that the *Lactobacillus* and *Bifidobacterium* strains used in probiotic products already in commercial use may affect the oral ecology in several ways including by killing pathogenic bacteria, activating the antimicrobial peroxidase system, specifically preventing the adherence of other bacteria and by modifying the protein composition of the salivary pellicle.

6. SUMMARY AND CONCLUSIONS

The aim of this study was to evaluate *in vitro* the oral colonisation potential and oral effects of the probiotic *Lactobacillus* and *Bifidobacterium* strains in commercial use. The main findings were as follows

1) There were significant differences in the *in vitro* studied oral colonisation potential between the probiotic strains. All strains survived in saliva, but they adhered differently to different surfaces. Furthermore, other oral bacteria influenced the binding of some strains. Individual differences were observed in the saliva-mediated effects studied.

2) All probiotic bacteria tested were acidogenic, although there were differences between the species in their use of different sugars and sugar alcohols. However, the acid production was strongly inhibited by the activated peroxidase system. Again, significant differences were observed between the strains studied in their sensitivity to the peroxidase system.

3) The results obtained suggest that probiotic bacteria used in commercial products may influence the oral microbiota by different, partly species or strain-specific means. These include the inhibition of adhesion, modification of the enamel pellicle, antimicrobial activity and activation of the peroxidase system.

The probiotic bacteria did not stand out as a group when compared to other lactobacilli or bifidobacteria used in this study regarding their binding to saliva-coated surfaces. Furthermore, not all of the observed saliva-mediated effects were specific to probiotic bacteria but properties common to several species. Thus, reference to previously observed probiotic properties, often suggested for gut health, is not adequate for arguing for the use of probiotic bacteria for oral health. However, species or strain-specific results were elicited as well. Thus, each probiotic strain needs to be evaluated separately for each effect of interest, and this is true, too, for the proposed health benefits in the oral cavity.

The salivary pellicle influences the colonisation of bacteria on the tooth surfaces and saliva is also a potential medium for a person-to-person transmission of bacteria, including pathogenic species. Thus, modifications in the salivary pellicle and the activation of a salivary defence system could influence not only oral but also general health.

Both potentially beneficial and potentially harmful effects of probiotic bacteria with respect to dental health were observed, but the significance of the perceived results needs to be further evaluated *in vivo*.

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