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LACTOBACILLUS REUTERI IN HEALTH AND DISEASE

MALMÖ UNIVERSITY

LACTOBACILLUS REUTERI IN HEALTH AND DISEASE

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GABRIELA SINKIEWICZ
LACTOBACILLUS REUTERI
IN HEALTH AND DISEASE

Malmö University, 2010
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Department of Biomedical Laboratory Sciences

*Dla Mamy i Taty i mojej Kochanej rodzinie: także Tym którzy już odeszli
i Tym którzy do niej wejdą!*

*Miłość jest jak nasionko leśne, z wiatrem szybko
leci, ale gdy drzewem w sercu wyrośnie, to tylko
chyba razem z sercem wyrwać je można.*

*-Henryk Sienkiewicz
Potop*

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ABSTRACT

People have exploited fermentation by lactobacilli for centuries as a means of preparing and preserving foods. Several different bacterial species are today used as probiotic, i.e. health promoting, bacteria in different products both for human and animal applications. By definition probiotic bacteria are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. The most commonly used bacteria for the probiotic concept are found within the lactic acid bacteria (LAB) group. One of several genera included in the LAB group is *Lactobacillus*. One species of *Lactobacillus*, *Lactobacillus reuteri* has been studied extensively and certain strains have indeed been shown to be probiotic with diverse beneficial effects, and thus it was challenging to further investigate the properties of these bacteria.

To put this thesis work into context, the field of probiotic research is described and examples of proven probiotic effects are discussed. The overall aim was to investigate *L. reuteri* and its microbial action in the microbiota of humans and its relationship to health and disease.

L. reuteri was shown to be indigenous in human milk. It was found in approximately one in seven nursing mothers living in geographically widely separated countries. Breast milk may be considered as a natural synbiotic and evidence from these results suggests that *L. reuteri* is one of the beneficial components in this regard. It was shown that *L. reuteri* supplementation of pregnant mothers and their offspring during the first year of life resulted in detection of *L. reuteri* in breast milk and infant stool. *L. reuteri* was also proven to be effective in reducing both gingivitis and dental plaque in patients with moderate to severe gingivitis, suggesting an improvement in periodontal health. Bacterial antagonism through the oral administration of the probiotic might have

contributed to the observed alleviation of symptoms and clinical manifestations of periodontal disease. Administration of *L. reuteri* resulted in the presence of *L. reuteri* in saliva, but no significant effect on supra- or subgingival microbiota was observed. The significant increase in plaque index in the control group with no significant change in the test group may however indicate a probiotic effect of *L. reuteri* in this study population of healthy individuals.

LIST OF PAPERS

- I. Occurrence of *Lactobacillus reuteri* in human breast milk. G. Sinkiewicz, L. Ljunggren, Microb Ecol Health Dis. 2008;20:122-126.
- II. Probiotic lactobacilli in breast milk and infant stool in relation to oral intake during the first year of life. T.R. Abrahamsson, G. Sinkiewicz, T. Jakobsson, M. Fredriksson, B. Björkstén, J Pediatr Gastroenterol Nutr. 2009;49:349-354.
- III. Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*. P. Krasse, B. Carlsson, C. Dahl, A. Paulsson, Å. Nilsson, G. Sinkiewicz, Swed Dent J. 2005;30:55-60.
- IV. Influence of dietary supplementation with *Lactobacillus reuteri* on the oral flora of healthy subjects. G. Sinkiewicz, S. Cronholm, L. Ljunggren, G. Dahlén, G. Bratthall, in revision, Swed Dent J, 2010.

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Contribution to the publications

In papers II and III, I took part in the planning process, was responsible for most of the experimental work, wrote the methodological parts of the papers and participated in analysing the data and in discussions concerning the manuscript. I performed most of the planning and experimental work in paper I, including analysing the data and I was the main writing contributor to the manuscript. In paper IV I took part of the planning and did most of the experimental work and was also one of the main writing contributors to the manuscript.

INTRODUCTION

History of probiotics

The art of preparing fermented milk products such as yoghurt goes back thousands of years. Elie Metchnikoff introduced the probiotic concept in the early 1900s by stating that “lactic bacilli are good for health” (1). It was at that time known that milk fermented with lactic-acid bacteria inhibits the growth of proteolytic bacteria because of the low pH produced by the fermentation of lactose. His colleague Henri Tissier found that bifidobacteria dominated the gut microbiota in breast-fed babies, and he later claimed that bifidobacteria restore the balance in the gut microbiota caused by diarrhoeal disease (2). The origin of the term “probiotic” is credited to Werner Kollath, who used “Probiotika” in a 1953 publication entitled “Nutrition and the tooth system” (3). The following year, another scientist, Ferdinand Vergin turned the focus more toward microorganisms in his article entitled “Anti-und Probiotika”, where the word “probiotic” appears to be a composite of the Latin preposition *pro* (“for”) and the Greek adjective *biotic*, the latter deriving from the noun *bios* (“life”) suggesting that the substance is beneficial to life and mainly contrasting it to antibiotics and their negative effects (4). In 1989 Roy Fuller emphasized that consumption of viable microbial cultures as dietary supplements will enhance one’s health and well being by improving one’s intestinal microbial balance (5). Since then, interest in probiotics has increased and the concept is now well established in microbiological research with a purpose to better understand what probiotics are and what they do. Many different definitions of the term probiotics have been proposed, but the currently used and most accepted is that of the Food and Agriculture Organization of the United Nations and the World Health Organization, that in 2001, in a Joint Expert Consultation, officially formulated it as “Probiotics (also called bacteria or cultures) are live microorganisms which when administered in adequate amounts confer a health benefit on the host”

(6). Most of the probiotics described belong to the *Lactobacillus* genus or the *Bifidobacterium* genus, but certain other bacterial strains may also be probiotic as well as the yeast *Saccharomyces boulardi* (7).

Probiotics do not permanently colonise the host and need to be regularly ingested for any persisting health promoting effects (8). Long-term intestinal colonization may only be possible immediately after birth and during very early infancy. Since probiotic bacteria, in general, are delivered in food, they must be resistant to the stressful conditions of the GI tract (9). The secretion of gastric acid constitutes a primary defence mechanism against most ingested microorganisms, therefore before reaching the intestinal tract, probiotic bacteria must first survive passage through the stomach and the digestive process as a whole (10). Thus, the bacteria are exposed to enzymes in the mouth, low pH in the stomach and bile in the upper intestine (9). Another important determinant of probiotic ability to e.g. modify host immune response is attachment to and growth in the gut epithelium (8).

Prebiotics and Synbiotics

By definition, prebiotics are selectively fermented constituents that allow specific changes, both in the composition and/or in the activity of the GI microbiota that confer benefits upon host wellbeing and health (11). Prebiotics trigger their effects mostly through the metabolism of the bacteria they promote, where prebiotic fermentation results in the production of increased amounts of carbon dioxide and of bacterial cell-mass. Effective prebiotics must not become digested in the upper gut, but manage to reach the large colon, and then be utilised by a group of microorganisms that have clearly identified health promoting properties. Low molecular weight carbohydrates, oligosaccharides in the fructo-oligosaccharide and galacto-oligosaccharide groups, are most likely to fulfil these criteria (8).

Synbiotics are mixtures containing both probiotic and prebiotic components. Survival of specific selected microorganisms is improved by stimulating their growth with the help of an additional supply of certain specific dietary fibres.

Health benefits of probiotics

The effectiveness of probiotics is strain specific and they contribute to host health through different mechanisms (suppression of production of virulence factors, prevention or inhibition of the proliferation of pathogens or modulation of the immune response). In addition, different species at varying densities populate different niches of the digestive tract. The GI microflora acts on its host mainly

by performing a variety of metabolic activities, protecting against colonization by pathogens, and priming and stimulating the gut immune system (8, 12). Some bacterial products, such as lipopolysaccharides, peptidoglycans, and lipoteichoic acids have immunomodulatory properties and contribute to the mucosal and systemic immunomodulating effects that ileal and colonic bacteria have in the host. Probiotics interact with the immune system at the level of cytokine production, mononuclear cell proliferation, macrophage phagocytosis, modulation of autoimmunity, and immunity to bacterial and protozoan pathogens (8). There is evidence that lactic acid bacteria may improve the immune system by increasing the number of IgA-producing cells as well as increasing the proportion of T lymphocytes and Natural Killer cells (13). Lactic bacteria have been found to modulate inflammatory conditions such as inflammatory bowel disease in adults and hypersensitivity responses such as milk allergies, an observation thought to be at least in part due to the regulation of cytokine function (14, 15). How probiotics counteract immune system over activity remains unclear, but a potential mechanism is the desensitization of T lymphocytes to proinflammatory stimuli (16).

Lactobacilli are often added to food to provide health benefits for the consumer. Requirements generally proposed for suitable probiotic strains are: human origin, acid and bile stability, the ability to adhere to intestinal cells and to colonise the intestinal tract, production of antimicrobial substances, antagonism to pathogenic bacteria, good growth *in vitro*, and safety in human use (17). These bacteria may even have therapeutic functions such as improved lactose utilization (as lactic acid bacteria actively convert lactose into lactic acid) and antimicrobial activity (competitive inhibition, i.e. by competing for growth), anticholesterol action (by breaking down bile in the gut and inhibiting its reabsorption), and anticarcinogenic activities (some strains have the ability to bind with heterocyclic amines, which are carcinogenic substances formed in cooked meat or by decreasing the activity of β -glucuronidase, which can generate carcinogens in the digestive system) (8, 9, 18). Adverse effects of LAB are rare and have made prophylactic use of probiotics common to prevent antibiotic-associated-, traveller's- and pediatric diarrhoea. Antibiotic-associated diarrhoea results from an imbalance in the colonic microbiota leading to changes in carbohydrate metabolism with decreased short-chain fatty acid absorption resulting in osmotic diarrhoea. Another consequence is overgrowth of potentially pathogenic organisms such as *Clostridium difficile* (19, 20). *Lactobacillus* probiosis can accelerate normalisation of the host's microbial

balance and thereby moderate acute episodes of diarrhoea. Probiotics are used to treat infections in the vaginal mucosa (8, 21) and lactic acid bacteria are also thought to aid in the treatment of *Helicobacter pylori* infections (22, 23). Additionally, probiotics were found to improve some symptoms of irritable bowel syndrome and to be effective in treating ulcerative colitis (24, 25). Lately, evidence that probiotics can also play a beneficial role in oral health has increased (26).

Probiotics are normally not associated with pathogenesis. Caution should be exercised when administering probiotic supplements to immunocompromised individuals or patients who have a weakened intestinal barrier. There have been some reported cases of bacteremia caused by lactobacilli, but this is a very rare event, promoted by severe underlying conditions such as serious gastrointestinal disorders. The long history of safe use of the bacteria in food fermentation remains the best proof of their safety (27, 28).

Lactic acid bacteria

Lactic acid bacteria (LAB) do not form a taxon, but the members are united because they share many characteristics. The most important genera of LAB are *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium*, *Tetragenococcus* and *Bifidobacterium*. They are Gram-positive, non-spore forming cocci and rods, which grow under microaerophilic to strictly anaerobic conditions. With the exception of bifidobacteria, all the genera of LAB usually show a G+C (guanine plus cytosine) content lower than 50 mol%, with respect to DNA base composition of the genome. Bifidobacteria are also considered as LAB because of similar physiological and biochemical properties, and also the sharing of common ecological niches such as the GI tract. LAB are divided into the homofermentative species that produce lactic acid, and the heterofermentative species that produce lactic acid together with ethanol and carbon dioxide. In humans the LAB are found in the oral cavity, the GI tract and the vagina. They are widespread in environments where carbohydrates are available, such as in milk and dairy products, fermented foods (meat products, olives, sauerkraut), fruits, and vegetables, and in the respiratory, gastrointestinal (GI) and genital tracts of humans and animals (12). Several metabolic compounds, e.g. carboxylic acids, fatty acids, hydrogen peroxide (oxidizes basic proteins), diacetyl (interacts with arginine binding proteins) and carbon dioxide (reduces membrane permeability) produced by LAB have antimicrobial effects (10). The carboxylic acids produced tend to lower the pH of

the gut (at least locally) and thus create a less desirable environment for harmful bacteria. LAB also produce bacteriocins which generally are antimicrobial oligopeptides of various composition and structure which act on closely related bacteria (affecting membranes, DNA- and protein-synthesis), while substances such as hydrogen peroxide affect gastric and intestinal pathogens and other microbes (29). Some bacteriocins act as signalling molecules in the interaction with other bacteria and with cells of the host organism (30).

The genus *Lactobacillus*

The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae*. The genus *Lactobacillus* includes 106 validly described species and is the most extensive genus in the order *Lactobacillales* (31). Lactobacilli are rods, normally present in the healthy GI and vaginal tracts, and are thought to be involved in the control and maintenance of the microbiota (32). In terms of numbers of bacteria found in faecal samples, the numbers of *Lactobacillus* are in the range of 5-8 log cfu/g (33). The concentrations of lactobacilli change with respect to both host age and dietary habits. These bacteria use carbohydrates as an energy source and produce lactic acid as the major metabolic end product.

Lactobacillus reuteri

A specific biotype of *Lactobacillus fermentum* (biotype IIb) was first isolated by Lerche and Reuter in 1962. In 1980, Kandler *et al.* described this biotype as *L. reuteri*, a new subspecies of heterofermentative lactobacilli, based on DNA-DNA homology (*L. reuteri* differs from *L. fermentum* in GC content, 39-41 mol% of its DNA, and has lysine as the peptidoglycan diaminoacid) (34). *L. reuteri* is a Gram-positive, non-sporeforming, non-motile, facultative anaerobic rod shaped bacillus, see figure 1. The optimum growth temperature is between 37-42 °C and the optimum growth pH is about 6.5 (no growth occurs below pH 4.5). *L. reuteri* does not require anaerobic conditions for growth and is normally cultivated in oxygen-limited atmospheres. *L. reuteri* strains are fastidious and rely on the availability of easily fermentable sugars, amino acids, vitamins and nucleotides. If these factors are provided, the organisms grow very fast, with duplication times of less than an hour, and *L. reuteri* can use several external electron acceptors (fructose, glycerol, nitrate, pyruvate, citrate and oxygen) to gain additional energy and increase growth rates even further (35). *L. reuteri* belongs to the group of obligate heterofermentative species that resides in the GI tract of humans and animals. It uses the phosphoketolase pathway for

fermentation (metabolic degradation) of carbohydrates to lactate, acetic acid, ethanol and carbon dioxide, see figure 2. This pathway has a poor energetic yield, which can be compensated for by using external electron acceptors. The presence of external electron acceptors, such as glycerol allows a heterofermenter like *L. reuteri* to gain an ATP from acetyl phosphate (acetyl phosphate + ADP $\Rightarrow$ acetate + ATP) rather than reducing (to generate NAD⁺) the acetyl phosphate to ethanol and consequently losing the energy transformation of the acyl phosphate high energy bond to the phosphoanhydride high energy bond of ATP, thereby providing greater ATP yields per mole substrate utilized, increased growth rates and higher biomass yields than obtained in the absence of glycerol (36). However, the *L. reuteri* strain ATCC 55730 was proven to use two functional glycolytic pathways. Adaptation to environments where external electron acceptors are present may lead to an efficient ATP/glucose ratio making the phosphoketolase pathway the dominant pathway. An active Embden-Meyerhof pathway, operating secondarily to the phosphoketolase pathway might adequately alleviate any intracellular redox burden in the *L. reuteri* strain ATCC 55730 (37).

Figure 1: *L. reuteri* visualised in different ways (from left to right): strain ATCC 55730 with magnification 10000x in scanning electron microscope, colony growth and appearance of different strains on MRS agar (upper left: mouse strain 4020, upper right: mouse strain 4000, lower left: chicken strain ATCC 55148, and lower right: turkey strain ATCC 55149), and mouse strains 4000 and 4020 colonizing the scamous region of the mice stomach with magnification 5000x in scanning electron microscope.

Mode of action

L. reuteri ATCC 55730 (and its daughter strain DSM 17938) has been demonstrated in several studies to have probiotic properties (32, 38-42). It is considered to be one of the few true indigenous *Lactobacillus* species in man (32). It has been shown to resist and survive in an environment with a pH as low as 2.5 and also survives in the presence of bile salts (43, 44). These experiments

were performed *in vitro* but most likely represent conditions similar to those found in the stomach and small intestine. A combination of different mechanisms, such as excretion of lactic, acetic and short-chain fatty acids, hydrogen peroxide and antimicrobial substances, and the ability to convert lactose to lactic acid, makes the species a successful growth inhibitor of pathogenic microorganisms. Lactic acid is produced as the racemic mixture L- and D-lactic acid. The broad-spectrum antimicrobial intermediary metabolite named reuterin is accumulated and secreted by *L. reuteri* under certain conditions (45). Reuterin is metabolized in a specialized bacterial compartment called the metabolosome (46). It is a low molecular weight, water-soluble, non-proteinaceous, and neutral end-product associated with the 1,3-propanediol fermentation of glycerol, and is a mixture of 3-hydroxypropionaldehydes (3-HPA), including a monomer, a hydrated monomer and a cyclic dimer. *In vitro* experiments have shown that it is formed during anaerobic growth by a two-step pathway in which glycerol is first dehydrated to form reuterin (a coenzyme cobalamin-dependent diol dehydratase catalyzes the conversion), some of which is then reduced to 1,3-propanediol (an NAD⁺-dependent oxidoreductase is responsible for this), a pathway proposed to regenerate NAD⁺ from NADH and to contribute to improved growth yield, see figure 2 (47). The ability to use 1,3-propanediol as an energy source might be an important colonization factor in the human colon, since easily accessible nutrients are in low supply due to their prior absorption in the small intestine. The enzyme for 1,3-propanediol utilization (diol dehydratase) is vitamin B₁₂ dependent and the encoding genes are organized in the same genomic context as the vitamin B₁₂ synthesis operon. The enzyme is also involved in the utilization of glycerol as an electron acceptor and in reuterin formation. This gene cluster is therefore likely to play several important roles in the biology of *L. reuteri* (46, 48). The presence of bacteria such as *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Staphylococcus*, *Clostridium*, and *Pseudomonas* stimulates the conversion of glycerol to reuterin (or increased release of reuterin from the cell), which acts locally and provides a competitive advantage in the intestinal tract (49). It is not known how much and when reuterin is produced in the gut since the aldehyde group of reuterin is very reactive and capable of spontaneous reactions with available amino-, sulfhydryl- and other functional groups, but a study using germ-free mice mono-associated with a reuterin producing strain of *L. reuteri* has demonstrated that reuterin can be produced by this bacterium *in vivo*, thus one can conclude that *in vitro* observations can be applied to *in vivo* situations (48). Concerning *in vivo* sources of glycerol for reuterin production, it is known that lipids are degraded throughout the GI tract, and also that both non-covalently and covalently bound lipids (neutral, glyco- and phospholipids) are found in

mucus. It is possible that host and/or microbial lipases generate glycerol for reuterin production from these and perhaps other lipid sources (50, 51). Reuterin can be synthesized by other heterofermenters (e.g. *L. brevis*, *Klebsiella* spp), but only as a transient metabolite that is immediately reduced to 1,3-propanediol. The ability to produce more 3-HPA than required to satisfy bioenergetic needs is unique in the GI tract to *L. reuteri* strains, and the excess is secreted, exerting potent antimicrobial activity in the surrounding microenvironment (52). Reuterin has proven bactericidal and bacteriostatic activity against a wide range of pathogenic bacteria, yeast, protozoa and viruses. Reuterin concentrations in the range of 15-30 µg per ml inhibit growth of Gram-negative and Gram-positive bacteria, yeasts, fungi and protozoa, while concentrations 4-5 times higher are required to kill lactic acid bacteria including *L. reuteri* itself, indicating that the antimicrobial activity of reuterin is of ecological and evolutionary significance (43, 47, 53, 54). The aldehyde group of reuterin is very reactive and inhibits bacterial growth by inducing oxidative stress in cells, most likely by modifying thiol groups in proteins and small molecules (47). Lactic acid and acetic acid, normally produced during normal carbohydrate degradation, also act as inhibitors of microbial growth and are especially potent against Gram-negative bacteria. *L. reuteri* has been observed to produce yet another low molecular weight antimicrobial substance, reutericyclin (a tetramic acid). It is an inhibitory compound that is structurally but not functionally related to naturally occurring tetramic acids, and is bacteriostatic or bacteriocidal to Gram-positive bacteria based on its activity as a proton-ionophore. Gram-negative bacteria and yeast are resistant to reutericyclin because of the barrier properties of their outer membrane (55).

Studies in birds and mice have shown the ability of *L. reuteri* to stimulate the development of ileal tissue, resulting in longer villi and deeper crypts (56). The intestinal surface growth may play a role in maintaining gut mucosal integrity and the larger surface area enhances the absorption of nutrients, minerals and vitamins, but the actual mechanisms behind the effect are unknown. One or more of the following may be involved- (a) *L. reuteri* induced cytokines affecting crypt cell mitotic activity, (b) *L. reuteri* induced production of defensins and other antimicrobial substances by the paneth cells in the crypts (thereby negating the growth-repressive effects of potential pathogens or other antagonists present in the gut) and (c) *L. reuteri* influence on the ability of other members of the gut microbiota to produce sufficient amounts of short-chain fatty acids (personal communication, Walter Dobrogosz).

Figure 2: Pathways for productions of reuterin (A) and heterofermentation of glucose (B) by *L. reuteri*.

Colonization

The mechanism of colonization/adherence in the GI tract, i.e. “how” this occurs is unknown. Colonization to the GI tract can be improved when *L. reuteri* is exposed to mucin (57). Mucin, the main component of the mucus secreted by the goblet cells will help *L. reuteri* to attach and replicate in the mucus layer over the epithelial cells in the GI tract and in the loose mucus layer in the lumen. Cell surface proteins involved in colonization and binding (adhesion) to mucus layers have been identified (57, 58). The gene corresponding to the mucus binding protein (Mub) has been identified in several *L. reuteri* strains and its presence correlates to a great extent with the adhesion of mucus *in vitro*. In addition, the existence of a collagen binding protein (CnBP) involved in adherence to epithelia and/or mucus has also been reported in *L. reuteri* along with extracellular glucosyltransferase enzymes (commonly named glucansucrases, GTFs) and inulosucrase (Inu) that synthesize different homopolysaccharides which contribute to cell aggregation and *in vitro* biofilm formation under acidic conditions (58, 59, 60). The glucans produced by *L. reuteri* may also act as prebiotics by stimulating the growth of probiotic strains or of beneficial strains of the GI tract. In such a scenario, *L. reuteri* will act as a good competitive exclusion agent against some pathogens.

Extracellular polysaccharides (exopolysaccharides, EPS) are synthesized either inside or outside the cell and secreted by a wide variety of bacteria including lactic acid bacteria, and are shown to contribute to dental biofilm formation and cell aggregation of streptococci (61). Bacterial EPS have a protective function in the natural environment against desiccation, phagocytosis and predation by protozoa, phage attack, antibiotics or toxic compounds and osmotic stress, but also they

play a roll in cell recognition, adhesion to surfaces and EPS will also facilitate the colonisation of various ecosystems (62). In the food processing industry, EPS from LAB can be used as viscosifying, stabilising, emulsifying, gelling, or water-binding agents. Many strains of lactobacilli produce homopolysaccharides (HoPS) (which in general are produced outside the cell) and oligosaccharides (OS) consisting of either glucose residues (glucans and gluco-oligosaccharides, GOS) or fructose residues (fructans and fructo-oligosaccharides, FOS) from sucrose by the extracellular enzymes glucosyltransferases and fructosyltransferases, respectively (63). Strains of *L. reuteri* produce glucans and fructans of different linkage types and express the *gtfA* and *inu* genes encoding a glucosyltransferase (GTFA) and an inulosucrase, respectively (64). Besides GTFA, two other GTFs from different *L. reuteri* strains have been characterized, GTF180 and GTFML1. These three enzymes are highly similar in terms of structures and amino acid sequences, but nevertheless synthesize different glucan products. The gene *gtfO* is present in *L. reuteri* ATCC 55730 and the homopolysaccharide and the corresponding enzyme in *L. reuteri* ATCC 55730 have also been characterised as a reuteran and reuteransucrase (GTFO), respectively (59). The concentrations of sucrose needed to yield significant glucan polymer production may normally not be achieved in the gut. In the oral cavity on the other hand, GTFO may contribute to polymer formation and colonization on oral surfaces. Some *L. reuteri* strains possess glucan-binding proteins that contribute to coaggregation, even though they do not produce glucan. Inu is a glucan-binding protein and a receptor for the glucan produced by GTFA (60).

Safety aspects

The safety (defined as absence of clinical adverse reactions such as nausea, stool characteristics, vomiting, flatulence or abdominal symptoms) of *L. reuteri* has been documented in several human clinical trials in healthy adults, children, infants and neonates as well as in immuno-suppressed HIV-positive volunteers (65-68). Two of these studies also showed good *in vivo* survival in humans confirmed by enumeration of *L. reuteri* in faecal samples. From these and other studies it was concluded that a dose of 10^8 cfu/day was well tolerated, safe and efficacious in man. A safety investigation was performed of the levels of D-lactic acid in the blood of infants at the age of 6 and 12 months as part of a prospective study of decreasing the risk of allergy. Very low levels of D-lactic acid were observed and no difference between the infants ingesting *L. reuteri* and those receiving placebo could be seen. The highest level observed was well within the normal range seen in humans (0.020 to 0.130 mmol/L) and no symptoms were reported that would normally be associated with acidosis (69, 70).

As shown for all other species of LAB, plasmids can be found in some strains of *L. reuteri*, and some of these plasmids have shown to encode antibiotic resistance markers (71-73). Heterofermentative lactobacilli are consistently resistant to vancomycin, but this resistance gene is intrinsic and is assumed to be non-transferable. There has been speculation as to whether probiotic strains can potentially act as reservoirs of antibiotic resistance genes. *L. reuteri* ATCC 55730 has been shown to possess a series of intrinsic antibiotic resistances. It does, however, carry specific unusual resistances to tetracycline and lincosamides as well as a β -lactam resistance that appears in approximately half of the members of this species (74). Lactobacilli added to the food chain must not carry transferable antibiotic genes according to the European Food Safety Authority. Consequently, the removal of two resistance plasmids against tetracycline and lincomycin in the commercial probiotic *L. reuteri* strain ATCC 55730, resulting in the *L. reuteri* daughter strain DSM 17938 without losing any probiotic characteristics was recently reported (75).

Health benefits

Several clinical studies have shown that *L. reuteri* ATCC 55730/DSM 17938 promotes health and improves defence against illness, such as GI tract problems (41), colicky symptoms in breast fed infants (40), reduction of infections (42, 76) and improved feeding tolerance in formula fed premature neonates (77). Orally administered *L. reuteri* has been shown to induce mucosal proinflammatory cytokines (TNF- α , IL-2 and IL-1 β) and thereby influence the immune response in mice (78). Several other studies have also demonstrated responses/regulations of macrophages, T- and B-cells, CD4+/CD8+ and antibodies of the gut associated lymphoid tissue (GALT) in both mice, poultry, and man (39, 52, 79). *L. reuteri* has proven effect in rats with acute liver failure in reducing bacterial translocation (transport of live bacteria from the intestinal lumen to the blood stream and internal organs of the body) (80). Translocation may be caused by bacterial pathogens, like *Salmonella* spp or by members of the normal gut microbiota when stress (starvation, trauma etc.) is applied to the host. The body's first line of defence against such translocation consists of the epithelial cells of the intestine, mucus produced by these cells and the intestinal bacteria naturally inhabiting the gastrointestinal tract. Immunological and lymphatic structures form a second line of defence. Moreover, another study has shown that *L. reuteri* has positive effects in maintaining gut mucosal integrity (81). *L. reuteri* administered to pigs and mice has been shown to decrease the level of serum cholesterol (82, 83). One possible mechanism behind this reduction of serum cholesterol is the ability

of the bacteria to produce bile salt hydrolase (BSH). Bile salt deconjugation is not solely beneficial to health, but can also carry pathological side effects, in such that excessive deconjugation can lead to malabsorption of lipids and lipid soluble vitamins and increased bile acid levels in the large bowel with enhanced risk for colon cancer (84, 85). Diarrhoeal diseases are one of the most common health problems encountered in childhood worldwide. During acute diarrhoea, the normal GI microbiota is radically changed, including decreases in *Lactobacillus*, *Bifidobacterium* and *Bacterioides* species. Prophylactic efficacy for community-acquired diarrhoea has been proven in 12 to 36 month-old children living in Mexico (86). Children receiving *L. reuteri* showed a significantly lower incidence of diarrhoea compared to untreated groups. Therapeutic effects for acute rotavirus-induced diarrhoea has been shown in hospitalized patients (age 6-36 months), where the duration of watery diarrhoea of the group receiving *L. reuteri* was reduced compared to the untreated group (41). A study where 40 dyspeptic adults infected with *Helicobacter pylori* received *L. reuteri* for 28 days, or placebo, showed that *L. reuteri* effectively suppressed the *H. pylori* infection and reduced the overall occurrence of dyspeptic symptoms, particularly abdominal distension, abnormal defecation and flatulence, without interference with the antibiotic treatment of the infection (23).

The evolution of the gut symbiont *L. reuteri* was characterized by population genetic structure and phylogeny (genetic relatedness over time) of strains isolated from six different hosts (human, mouse, rat, pig, chicken and turkey) from widespread geographic locations and revealed considerable genetic heterogeneity within the *L. reuteri* population as a whole but a remarkably conserved genetic makeup in strains from the same host species. This indicates a long evolutionary association, a stable relationship and the presence of ecological symbiotic strategies in this species with particular vertebrate host species, i.e. a highly specialized symbiosis between this microbe and its host and the development of mutualistic interactions. The population structure also indicated that *L. reuteri* lineages evolved with groups of related vertebrates (rodents and poultry), i.e. suggesting occasional horizontal transfer between these hosts (87). High host specificity, reflected by phenotypic characteristics of strains and the phylogenetic distribution of these phenotypic factors indicate that they evolved to access specialized niches in the particular hosts, and that the host environment is the major factor in the evolution of *L. reuteri* (54).

Human microbiota

People are generally healthy and show no symptoms of infection even though they live in symbiosis with a wide variety of microorganisms. The human organism (the host) coevolved with a normal microbiota over millennia and developed mechanisms that monitor and control this microbial ecosystem. There are more than 2000 species of commensal bacterial organisms within our bodies, the vast majority in the gut, and only about 100 known species of pathogens (88). These commensals (a word derived from the Latin *con mensa* meaning “sharing a table”) help the host keep the numbers of pathogenic and potentially pathogenic species at levels that will cause no harm. In humans it is estimated that microbiota account for 1.5 kg of biomass in the GI tract (89). The skin is also a large reservoir for bacteria whereas smaller numbers are found in the lungs, the mouth, the nasal cavity, and in the vagina. An adult human is made up of approximately 10^{13} cells but serves as a host for approximately 10^{14} bacteria (90).

The oral cavity and its microbiota

The mouth includes the tongue, palate, buccal and lingual mucosa, teeth and plaque (both supragingival (above the gum line) and subgingival (below the gum line)), all of which are bathed in saliva. During and shortly after birth the epithelial surfaces in the oral cavity become colonized by various species of the indigenous microbiota that tend to persist in the mouth, which may play a role in the competition with other bacteria and prevent the growth of those that may colonize later (91). The microbiota of the mouth is highly complex, containing a wide variety of bacterial species that differ within and across specific locations. Key organisms include *Streptococcus* species (particularly *S. sanguinis*, *S. mitis* and *S. crista*), *Lactobacillus* species (particularly *L. gasseri*, *L. fermentum* and *L. salivarius*), *Fusobacterium*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Neisseria*, *Veillonella*, *Corynebacterium*, *Actinomyces* and *Treponema* species. It is estimated that about 700 species are carried in the mouth and oropharynx, and under normal conditions bacterial counts in saliva are 10^6 to 10^8 cfu per millilitre, with *Lactobacillus* spp. constituting only a small part of the oral microbiota. In the mouth, bacteria have to resist the oral environmental conditions and defence factors present in saliva and unless they adhere to oral surfaces the bacteria are rapidly swallowed. Bacteria can attach to immobilized salivary proteins (i.e. to acquired dental pellicle), attach to epithelial cells or (co)aggregate with other bacteria already present.

The most common types of oral disease, dental caries and periodontal disease, are both related to dental plaque and seem to occur when the normal balance between the microorganisms and the host is disturbed in some way. Unbalanced oral microbiota can be associated with serious systemic diseases such as spontaneous preterm births, coronary heart disease, atherosclerosis and chronic kidney disease (92). Dental caries is usually associated with increased numbers of mutans streptococci at the sites of disease. The production of carboxylic acids from dietary sugars (sucrose, fructose and glucose) is a key factor in the caries process. The *Lactobacillus* group contains homo- and heterofermentative species and are all aciduric (can withstand a pH as low as 3.5) and the low pH generated from acids challenges the homeostasis in the oral microbial community, and are thus traditionally associated with the development of dental caries.

Periodontitis is an infectious disease, affecting at least one-third of the population worldwide and is defined as a plaque-dependent disease that begins as gingivitis (inflammation of the gums) which is associated with poor oral hygiene and which affects the majority of the adult population with increased prevalence with increasing age (the most pronounced manifestation occur in middle-aged patient groups). According to WHO surveys, most children have signs of gingivitis and among adults the initial stages of periodontal disease are highly prevalent. Not all gingivitis lesions progress to periodontitis. In humans periodontitis is predominantly associated with an inflammatory response where the crevicular fluid increases (the crevice enlarges to become a pocket entered by components of the immune system and different glycoproteins) and an accumulation of Gram-negative strictly anaerobic microorganisms in the dental plaque occurs. Over time, the infection and inflammation may spread from the gingiva to the ligaments and supporting bone to cause destruction of this connective tissue attachment of the teeth. However, it has to date not been possible to identify a single bacterial organism as the causative agent in the etiology of periodontitis, but evidence points to potential candidates (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* have most frequently been reported as significant periodontopathogens) influencing pathogenesis which may be considered as risk indicators. Bacterial endotoxins together with some metabolic by-products produced by periodontal pathogens lead to severe damage of periodontal tissue components (92).

The mechanism of action of probiotics in the oral cavity is not fully understood. Action may be based upon replacement or competitive exclusion of members of the microbial community, through competition with harmful organisms by occupying their niche, or by restricting the adhesion capability of pathogens to surfaces by modifying the protein composition of the pellicle (degradation of salivary proteins). Further mechanisms may include negatively influencing the vitality or growth of the microbiota by competition for essential nutrients, through pH changes in the environment and through degradation of virulence factors of the pathogens. Further, co-aggregation or cooperation within the normal microbial community and maintenance of the microecological balance and/or immunomodulation (locally and/or systemically) may be involved. Co-aggregation is a key mechanism in biofilm formation and a significant factor in dental plaque development. Interspecies adhesion is mediated via protein/glycoprotein-carbohydrate cell-surface interactions such as the activity of GTFA enzymes, surface protein antigen (Pac), glucan-binding protein C and dextranase (93). Immunity is modulated by, for example, alteration of the balance of pro-inflammatory and anti-inflammatory cytokines secreted by epithelial cells. Lactobacilli can produce lactic acid, hydrogen peroxide and bacteriocins or bacteriocin-like substances with an inhibitory activity against a wide range of bacterial species, including oral streptococci (94).

The metabolic activity differs between various probiotic strains and it was shown in an *in vitro* study that the *L. reuteri* strain ATCC PTA 5289 was almost inactive for different dietary sugars under both aerobic and anaerobic conditions, and may not increase the risk of dental caries. Hence, not all *Lactobacillus* spp have a caries-inducing effect (95). It has been shown that consumption of yoghurt containing *L. reuteri* ATCC 55730 reduced oral carriage of *Streptococcus mutans* and that acids from *L. reuteri* had negligible effects on calcium release from the enamel (96). A number of studies describe similar results from *L. reuteri* ATCC 55730 and ATCC PTA 5289 on the levels of salivary mutans streptococci and lactobacilli with a reduction of mutans streptococci levels (97-99). Although the suppressive effect on cariogenic microbiota has been demonstrated, the survival of *L. reuteri* in the oral cavity is still undocumented. According to Haukioja *et al.*, a bacterium needs to adhere to oral surfaces (mucosa and dental tissues) as a part of the oral biofilm to be able to have a probiotic effect (through competition with the growth of cariogenic bacteria and/or periodontal pathogens) (100). Yli-Knuuttila speculated that colonization of oral probiotics is rather temporary and that the chance of a permanent colonization of exogenous/probiotic bacteria is

only possible in childhood (101). It has been shown that vaginally born children who are exposed to more maternal bacteria at birth than children born via caesarean section are colonized later in life by cariogenic bacteria, which may be explained by a competition between lactobacilli and streptococci. It might be possible that a regular intake of probiotic bacteria during early childhood when the GI biota is established, increases the chance of colonization and may decrease the incidence of dental caries in children (102, 103).

Research on potential beneficial effects of probiotic supplementation on periodontal disease is in its infancy, but has already delivered some promising results. The hypothesis has been that the bio burden of periodontal and gingival pathogens could be regulated by means of antagonistic interactions, so called "replacement therapy" thereby controlling the progression of the disease. Köll-Klais reported that resident lactobacilli biota inhibited the growth of *Porphyromonas gingivalis* and *Prevotella intermedia* *in vitro* (104). Twetman *et al.* showed a significant reduction of clinical signs of gingivitis (bleeding on probing, gingival crevicular fluid (GCF)) as well as a reduction of pro-inflammatory mediators such as TNF- α , IL-8, and IL-1 β by *L. reuteri* ATCC 55730 and ATCC PTA 5289 in healthy adults with moderate gingivitis. It was concluded that the reduction of pro-inflammatory cytokines in GCF might be proof of the probiotic approach to gingival inflammation (105). Mayanagi *et al.* reported that some periodontopathic bacteria were reduced by a probiotic strain of *L. salivarius* (106). A clinical trial showed that *L. salivarius* WB21 decreased plaque index and pocket probing depth as well as reducing the prevalence of periodontal pathogens (107). In an *in vitro* study by Van Hoogmoed *et al.* the aim was to identify bacterial strains that reduce adhesion of periopathogens to surfaces with the hypothesis that changing adhesion properties would develop a healthier microbiota. Different streptococci, *Actinomyces naeslundii* and *Haemophilus parainfluenzae* were shown to inhibit the adhesion of *P. gingivalis* in this model (108).

Microbial ecology of the gastrointestinal tract

The composition of species in the GI tract is unique for the individual host, and the pattern is established within the first years of life and depends to some extent on genetic factors but predominately on mode of delivery at birth, food intake, ingested bacteria and other environmental factors. However, the same main genera of bacteria are present in all individuals. Under normal conditions the composition of the microbiota is relatively stable, although an increase in

Clostridium spp and a decrease in bifidobacteria occur with age. Bacterial counts are low in the upper GI tract due to high acidity (pH about 2) and short transit time from mouth to ileum (about 4-6 h). The human stomach contains 0 to 10^3 cfu/ml gastric juice, a number that increases in the small intestine (10^3 to 10^5 cfu/ml) before rising even more in the large bowel. There are 500 to 1000 different species of bacteria with a population level of 10^{11} to 10^{12} viable cells per gram of intestinal content in the colon, where the most common bacterial groups present are bifidobacteria, eubacteria, clostridia, fusobacteria, streptococci, enterococci, lactobacilli, and the dominating bacteroides (89). The transit time in the colon is slow, about 55 hours, and microbial colonization is thus facilitated (109). *Lactobacillus* strains are found in every part of the GI tract, but are not the predominating genus in the colonic mucosa (110). Most of the microbiota in the GI tract is located in the intestinal lumen or in the loose mucus close to the lumen without direct contact with the epithelium (mucosa) and its constituent cells (enterocytes) (111). The colonic microflora acts as a barrier to foreign organisms, but nevertheless pathogens can become established when the microbial integrity is disturbed through illness, physiological alterations in the gut, or antibiotics treatment (8, 12, 112).

Intestinal microbiota of infants

All humans are normally born germ free, thus without any microbes on either the exterior or the interior surface of the body. Immediately at birth, any newborn is exposed to a variety of different microorganisms, that colonize the skin, respiratory tract and GI tract and the microbes first to establish themselves have abundant space and nutritional components available. As space and nutrients become less abundant, the fittest microbes occupy all niches, and eventually, a complex ecosystem within the host is established.

Human breast milk is considered to be of primary importance for the optimal health, growth and development of the infant (113). Aside from its nutritional value, it also contains several bioactive factors (immunological factors such as immunoglobulin A and lysozyme) that may enhance the infants' defences (18, 113) and protect it against colonizing pathogens (114, 115). It has been suggested that commensal and potential probiotic bacterial components present in breast milk may also be involved in the development of the infant's gastrointestinal mucosal tissues and its acquisition of a healthy gut microbiota (18, 116). Mothers' milk is a consistent source of microorganisms for the neonatal gut during several weeks after birth, and it is only after the introduction of solid

food that the gut microbiota become more diverse and begin to resemble that of adults (116). The bacterial species generally found in healthy human breast milk belong to the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Micrococcus*, *Enterococcus* and *Bifidobacterium* (115-117). Some of these genera originate from the surface of the nipple and the surrounding skin and may be adapted to survival in the milk ducts in the breast (113, 116). The importance of diet on bacterial colonization of the gut has been explored and breastfed children are dominated by bifidobacteria and lactobacilli while formula-fed children have more bacteroides, clostridia and enterobacteriaceae (118).

It has been observed that fewer Swedish infants are colonised by *Lactobacillus* than Estonian infants (119). This has been attributed to differences in lifestyle, as for example eating habits and excessive hygiene levels. People with an anthroposophic lifestyle, consume a diet comprising vegetables fermented usually by lactobacilli and an anthroposophic lifestyle has been shown, for example, to reduce the risk for allergy in children (120, 121). An interesting probiotic effect is the ability to reduce symptoms of atopic disease. Björkstén *et al.* have shown that atopic children have a different microbial community structure than healthy children, where the level of lactobacilli and bifidobacteria is reduced. Increased incidence of allergies may be due to decreased microbial pressure in early childhood (122). Administering the *L. rhamnosus* strain GG to pregnant mothers, and to the children themselves for 6 months, reduced atopic disease among the children, examined at 2 and 4 years of age (123, 124). Another study where children with moderate or severe atopic symptoms were supplied with *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 showed decreased severity of eczema after 6 weeks of consumption (125). The intestinal microbiota is the major driving force in maturation of the immune system after birth and these trials are based on the hypothesis that by probiosis, the infant's immature immune system is triggered in a direction that favours tolerance to common antigens in the environment.

AIMS OF THE THESIS

L. reuteri strains have been shown to have diverse probiotic properties and thus it seemed appropriate to further investigate the characteristics of this bacterium. The main theme of this thesis was to study *L. reuteri* and its microbial action in the microbiota of humans and its relationship to health and disease.

The specific aims of each individual paper were:

Paper I: to investigate the occurrence of *L. reuteri* in human breast milk and the possible links between the presence of *L. reuteri* and the geographic areas of residence of the donating mothers.

Paper II: to assess the prevalence of *L. reuteri* in stool and breast milk from mothers and their infants after supplementation with *L. reuteri*, to evaluate factors influencing the levels of *L. reuteri*, and to assess the influence on certain aspects of microbial ecology that may be associated with allergy.

Paper III: to investigate if *L. reuteri* could be effective in the treatment of gingivitis and further to evaluate the influence of this probiotic on plaque and the lactobacilli population in the saliva.

Paper IV: to investigate the presence of *L. reuteri* in saliva after supplementation with *L. reuteri* and the probiotic effect of *L. reuteri* on plaque index and the supra- and subgingival microbiota.

MATERIALS AND METHODS

BACTERIAL MEDIA

Modified Man Rogosa Sharpe (MRS) media

So-called MRS agar was developed by de Man, Rogosa and Sharpe in 1960 and the thus prepared medium is transparent and light amber in colour. Generally, the properties of the medium is very important for the appearance of typical colonies and for enhancing visual differences in colony morphology. This particular medium allows the cultivation of lactobacilli and captures essentially the whole group of lactic acid bacteria, but in its original version, is not selective. Occasionally unwanted *Streptococcus* or lactococci will grow as a thin "lawn" or as "pin point" colonies. Distinct differences in colony morphology, however, allow for identification and accurate enumeration.

The addition of the reducing agent cysteine hydrochloride reduces the amount of oxygen available to the growing colonies. Since most *Lactobacillus* prefers little or no oxygen, this optimizes growth. In the case of mixed culture evaluations, such as from faecal samples, the colonies are more differentiated in the MRS-cysteine plate than in the regular MRS plate.

Improved selectivity can be obtained by the addition of 2% sodium acetate (w:v) to MRS agar to create a selective pH of 5.3, which makes this medium suitable for the enumeration of *Lactobacillus* and *L. reuteri*. Sodium acetate addition results in an increased inhibition of Gram-negative bacteria and restricts the colony size of other genera. Growth of *Enterococcus* and *Streptococcus* will appear as small pin point colonies, whereas *Lactobacillus* grows as larger colonies even on crowded plates. Colonies become larger and larger as the dilution increases, mainly because there is less competition for nutrients.

Nevertheless, the colony morphology, and also the colour is maintained which allows for differential enumeration. This medium supports good growth and better *L. reuteri* identification by the overlay method.

For faecal sample evaluation in the presence of other lactic acid bacteria, MRS with 2% sodium acetate (w:v) and 50 mg/L vancomycin is a very selective medium, since only bacteria resistant to vancomycin will grow. These additions to the medium entirely eliminate the *Streptococcus* as well as some lactic acid bacteria (*L. casei*, *L. acidophilus*, *L. bulgaricus*, *Bifidobacterium*), and hence, this medium cannot be used to evaluate total *Lactobacillus*. However, the *L. reuteri* colony count is facilitated.

The ability to grow on MRS agar supplemented with 2 mg/L ampicillin differs between the *L. reuteri* strains ATCC 55730 and ATCC PTA 5289, with ATCC 55730 growing well whilst ATCC PTA 5289 which lacks the intrinsic penicillin resistance present in 55730, is inhibited. After incubation, cfu's were enumerated on the MRS-amp and on the MRS sodium acetate and vancomycin plates. Subtraction of cfu's on the MRS-amp plate from those on the modified MRS plate corresponds to the count of ATCC PTA 5289.

***Lactobacillus* selective medium (LBS)**

LBS agar is a very selective medium. By adding 1.3 ml glacial acetic acid per litre, the plates reach a pH of around 5.7. *Enterococcus* do not normally grow on LBS but *Lactobacillus* in general do. However, LBS also restricts the growth of *Lactobacillus* and sometimes may present a hindrance in the identification of *L. reuteri* by the overlay method.

DP medium

This medium prepared by the addition of 2 mg/L dicloxacillin, 0.5% propionic acid, and cysteine hydrochloride to Colombia agar with adjustment to pH 6.8, is used for the enumeration of *Bifidobacteria*.

***Clostridium difficile* media**

Samples were diluted in 0.05% yeast extract solution containing oxyrase and plated on *Clostridium difficile* selective (CDSA) agar, a selective and differential medium for the primary isolation of *C. difficile* from faecal specimens. Cefoxitin and cycloserine are incorporated to inhibit the normal faecal biota whilst oxyrase is an enzyme additive used to provide anaerobic conditions. Oxyrase prevents

oxygen intrusion into the medium and further removes oxygen from the trapped air inside tubes or bottles.

Lactobacilli carrying medium (LCM)

LCM broth with added arabinose and ribose as carbohydrate sources, will enrich *Lactobacillus* species that utilize these particulate sugars as is the case of *L. reuteri*. Most organisms utilize dextrose and this medium thus becomes selective through the lack of dextrose.

Colony forming units (cfu)

A viable cell with the ability to divide (form offspring) can yield one colony. The viable count or colony count is the number of cells in the sample capable of forming colonies on suitable agar medium. Aggregates of two or more cells form only a single colony, so aggregation of cells may cause an erroneously low viable count. Viable counts are expressed as the number of colony-forming units, rather than as the number of viable cells (since a colony forming unit may contain one or more cells).

Colony morphology characteristics

Colonies of *Lactobacillus* should be apparent within 2-3 days of incubation at 37 °C. Colour, density and surface characteristics give *L. reuteri* a typical colony appearance that allows for a consistent and clear-cut differentiation from, for example, *L. acidophilus*. *L. reuteri* colonies have a perfectly circular, convex, white opaque ("milky"), glistening "fried-egg" like appearance and usually form the largest colonies on the plate (2-4 mm in diameter), whilst *L. acidophilus* will form smaller colonies that are flat, translucent white, and with irregular edges. *L. casei* will form round, convex, white colonies with a typical Gram stain of stacked, slender Gram-positive rods. *Bifidobacterium* colonies will be small, circular, convex shaped with a smooth outer edge and with a typical Chinese letter configuration in the Gram stain (V-, X- or Y-shaped Gram-positive rods). *L. bulgaricus* colonies are large, grainy looking, and dry, with very long twisted and intertwined Gram-positive rods in the Gram stain. *Streptococcus* spp colonies are small, white and round, and are typical Gram-positive cocci with the Gram stain.

Criteria for enumeration

The only true danger in using only MRS with sodium acetate and vancomycin and LBS lies in the fact that since both are selective, sometimes the total *Lactobacillus* reported may be less than that actually present. As a rule, "total *Lactobacillus*

cfu” is calculated as the average of the ”*Lactobacillus*” count in LBS medium. The *L. reuteri* cfu is calculated as the average of the ”reuterin positive” colony count in each medium.

Copacabana method for spreading colonies

Sterile glass beads (Ø 4 mm) for the dispersion of bacteria on solid media were used, which avoids smeared colonies or damage to the agar. For plating, 6-8 glass beads were shaken onto the plate. The plate was then agitated with a random shaking motion so that the glass beads roll over the entire surface of the plate and a uniformity of spreading over the plate is achieved. Once the liquid is absorbed, the plate is inverted and the beads discarded. This allows for faster plating and generates more distinct colonies on the plate (126).

Overlay technique for the detection of *L. reuteri*

L. reuteri produces ”reuterin” in the presence of glycerol. The bacteria were grown for 48-72 h on MRS agar plates in an anaerobic atmosphere. For enumeration, it is better to choose plates containing outgrown colonies, which are not too crowded (i.e. maximally about 100-150 colonies). The plates were then overlaid with 200 mM glycerol agar (1% agar) and incubated at 37 °C for 30-45 min. Reuterin was detected by the addition of 5 mL 2,4-dinitrophenylhydrazine solution (0.1% in 2 M HCl). After 5 min incubation, the solution was discarded and 5 mL 5 M potassium hydroxide solution was added. Reddish brown colour development around the colonies demonstrates the presence of reuterin and thus *L. reuteri* (53).

Replica plating technique

This technique enables repetitive transfer of isolates from one substrate to other agar media for further classification. Replica marks are placed on the back of the plates and the top and bottom of the plates are marked with a pen (in order to orientate the plates in an identical manners for reading). A velveteen square is placed on a wooden cylinder and held firmly in place. Slight pressure is applied to the velveteen cloth on the agar surface in the initial plate. The imprint of growth is transferred to an MRS sodium acetate and vancomycin plate and also to an MRS-amp plate. The imprinted fabric provides the pattern of colonies to subsequent plates. The plates are incubated anaerobically at 37 °C for 48-72 hours followed by enumeration (127).

Randomly amplified polymorphic DNA-PCR technique

For grouping and typing, *L. reuteri* reuterin positive isolates were randomly selected from the MRS medium, purified by streak plating and subjected to sequence analysis of 16S rDNA, performed as described by Magnusson *et al.* (128). Bacterial DNA was isolated from bacteria grown in MRS broth using the DNeasy™ Tissue Kit (Qiagen). The PCRs were performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare). Bacterial DNA (0.5 µL) was added to the PCR mix (in total 25 µL) and the 16S rDNA was amplified by running the program (94 °C for 30 s, 54 °C for 30 s, 72 °C for 80 s, step 1-3 30 cycles, 72 °C for 10 min, 4 °C for ∞) using primers 16S.S (5'-AGAGTTTGATCCTGGCTC-3'; position 8-25 in *E. coli* 16S rRNA) and 16S.R (5'-CGGGAACGTATTCACCG-3'; position 1385-1369 in *E. coli* 16S rRNA). The PCR products were separated by using standard agarose gel electrophoresis and stained with ethidium bromide.

RESULTS AND DISCUSSION

Paper I- Occurrence of *Lactobacillus reuteri* in human breast milk

A global epidemiological study regarding the presence of *Lactobacillus* generally, and *L. reuteri* specifically, showed that 15% of 220 women in Sweden, Denmark, Israel, South Africa, South Korea, Japan and Peru had *L. reuteri* in their breast milk. Further, 50% of mothers from rural areas in Japan and Sweden were *L. reuteri* positive, whereas mothers from urban areas in South Africa, Israel and Denmark had very low or non-detectable levels. There were no significant differences in the prevalence of total *Lactobacillus* or *L. reuteri* in women from rural and urban habitats in the participating countries.

This study suggests that *L. reuteri* is a natural component of human milk. *L. reuteri* was found in approximately one in seven nursing mothers living in geographically widely separated countries. Breast milk may be considered as a natural synbiotic and evidence from this study suggests that *L. reuteri* is one of the beneficial components in this regard. It is well known that breast milk colonizes a baby's intestine with healthy bacteria that will help the baby fight infections. Recently, Wang *et al.* have discovered how *L. reuteri* found in breast milk reduces or eliminates painful cramping in the gut. As previously stated this bacterium naturally occurs in the gut of many mammals and can be found in human breast milk as we have demonstrated. This discovery suggests that an increased intake of this bacterium may help alleviate symptoms of a wide range of gut disorders, such as irritable bowel syndrome, inflammatory bowel disease, functional bowel disorders, and constipation (129).

Paper II- Probiotic lactobacilli in breast milk and infant stool in relation to oral intake during the first year of life

In this prospective, randomised, double blind, placebo-controlled study, 232 women were randomly assigned to take either *L. reuteri* or placebo for the last 4 weeks of their pregnancy. Their babies continued with the same study product and were studied from birth until 12 months of age. The prevalence of *L. reuteri* was higher in the faeces of infants whose mothers had taken the active supplement compared to those whose mothers had taken placebo. The highest prevalence was recorded at 5-6 days of age (82% in supplemented vs. 20% in placebo; $p < 0.001$). The first expressed breast milk (colostrum) was positive for *L. reuteri* in 12% of the mothers given *L. reuteri* and in 2% of mothers in the placebo group. *L. reuteri* supplementation did not appear to affect bifidobacteria or *C. difficile* colonisation. In conclusion, *L. reuteri* can be detected in breast milk after oral supplementation to the mother and in almost all infants after oral supplementation during the first year of life.

Both *L. reuteri* and Bifidobacteria are natural habitants of the GI tract (33, 130). In general, lactobacilli have been observed in the stomach, jejunum and ileum (small intestine) and in the colon. Bifidobacteria colonize the same locations with the exception of the stomach. Faecal counts of *Lactobacillus* are in the range of 5-8 log cfu/g, whereas bifidobacteria are in the range of 8-10 log cfu/g (33). The concentrations of lactobacilli and bifidobacteria (as well as other natural habitants) changes with respect to age and dietary habits. In summary, the number of bifidobacteria present in the healthy human gut is approximately two orders of magnitude higher compared to lactobacilli, which concords well with this study result.

Consumption of *L. reuteri* had no effect on total *Lactobacillus* spp. enumerated from the faeces. Among the metabolites produced by *L. reuteri* are bacteriocins, and some bacteriocins inhibit the growth of other bacteria that belong to similar species or other genera. Thus, the administration of *L. reuteri* could lead to a replacement of other lactobacilli in the human GI tract. The prevalence of *L. reuteri* declined after the first week of life despite continuous supplementation and a high compliance. Possible explanations include competition from other bacterial species, a lower proportion of bacteria surviving passage through the stomach due to decreasing pH levels with age, and immune recognition (IgA-mediated mechanism) of *L. reuteri* resulting in an increased shedding of the bacteria.

Paper III- Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*

After 2 weeks of a twice-daily intake of a probiotic chewing gum, the gingival index fell significantly in all groups. Two different strains of *L. reuteri* were studied, designated Lr-1 (ATCC 55730) and Lr-2 (ATCC PTA 5289). The Lr-1 group, but not the Lr-2 group improved more significantly than placebo ($p < 0.0001$). Plaque index fell significantly in both *L. reuteri* groups between days 0 and 14, with no significant change in the placebo group. Both strains colonized the saliva, Lr-1 in 65% of patients and Lr-2 in 95% of patients.

L. reuteri was proven to be effective in reducing both gingivitis and dental plaque in patients with moderate to severe gingivitis, suggesting an improvement in periodontal health. The mechanism of action of *L. reuteri* might be related to the production of anti-microbial substances or an anti-inflammatory effect through the inhibition of pro-inflammatory cytokines. Bacterial antagonism through the probiotic administration in the oral cavity might also contribute to the observed alleviation of symptoms and clinical manifestations of periodontal disease.

The administration of these probiotics by means of a chewing gum could provide prolonged contact with oral tissues and facilitate probiotic adhesion to saliva-coated surfaces. Saliva mediates bacterial adherence to oral surfaces and saliva samples have often been used to evaluate microbial composition in the oral cavity. However, saliva microbial composition may underestimate the true situation in the oral biofilm. Motisuki *et al.* reported that the most suitable method to detect *Lactobacillus* spp. levels in the oral cavity is the stimulated whole saliva (131), but it may be more relevant to study saliva together with the supra- and subgingival occurrence of indigenous and exogenous bacteria.

Paper IV- Influence of dietary supplementation with *Lactobacillus reuteri* on the oral flora of healthy subjects

In this study, where 23 healthy individuals were supplemented with a chewing gum containing *L. reuteri* ATCC 55730 and ATCC PTA 5289 or placebo for 12 weeks, a significant increase in total *Lactobacillus* counts was observed in the saliva in both groups ($p < 0.05$) and a significant increase of *L. reuteri* was seen only in the test group ($p < 0.008$). The termination of intervention resulted in a wash out of *L. reuteri*. The control group showed a significant increase in plaque index after 12 weeks ($p < 0.023$) whilst there was no significant change in the test group. A significant increase was found for most bacterial species in both

groups in supra- and subgingival plaque with no significant difference for any of the species between the groups. The ratio between "bad/good" supragingival bacteria decreased for the test group but this decrease did not reach significance ($p < 0.08$ compared to $p = 0.52$ in the control group). The corresponding ratio for subgingival bacteria decreased significantly in both groups.

The increase in plaque index in the control group might suggest that *L. reuteri* inhibited plaque build up, as shown in paper III where plaque index decreased significantly in the two *L. reuteri* groups. The occurrence of *L. reuteri* in saliva decreased after the end of the intervention period, indicating that no permanent colonization had occurred and that oral persistence of *L. reuteri* was only temporary. This is in agreement with previous findings, where *L. reuteri* was not detected in any of the subjects after 5 weeks post-treatment (132). Individual responses, underlined by host-dependent factors such as age, determine colonization in general. It is questionable whether a permanent installation can occur in healthy adults where a preestablished microbiota is present, or where the environmental prerequisites for a more permanent colonization and establishment of *L. reuteri* in the oral cavity might not be present. Therefore, continuous administration should be maintained for permanent colonization and beneficial effects. Microbiologically no significant probiotic effect could be found, although there was a tendency towards a healthier microbiota in the *L. reuteri* group.

In a study carried out by Kõll-Klais *et al.* lactobacilli could not be found in subgingival samples in any of the patients with chronic periodontitis, demonstrating that the subgingival region might not be a common habitat for lactobacilli (104). Haukioja *et al.* tested binding of different *Lactobacillus* strains *in vitro* and found that *L. reuteri* ATCC 55730 binds to a low degree to saliva-coated microtitre wells, hydroxyapatite beads and BSA-coated hydroxyapatite beads. Survival in saliva was on the other hand high (100). These results were also confirmed by a study of Jones & Versalovic, where the *L. reuteri* strain PTA 5289 was more adherent than ATCC 55730 and differed with respect to ability to form biofilms (adherent structured microbial communities). In contrast the *L. reuteri* strain ATCC 55730 produced greater quantities of reuterin than ATCC PTA 5289. Probiotics in biofilm-like communities may be essential for long-term colonization and persistence of the microbiome, while reuterin may be important for maintaining a healthy microbiota by preventing overgrowth by other commensal and pathogenic microorganisms (133). The two strains used

in the study (the *L. reuteri* strain ATCC 55730 is derived from mother's milk while PTA 5289 is an oral isolate) have different properties and were specifically selected for their synergistic properties in reducing the oral bacteria associated with dental plaque, gingivitis, and caries. The distribution ratio in cfu between ATCC 55730 and ATCC PTA 5289 in the saliva samples was 1:4.

The complexity of the periodontal microbiota resembles that of the GI tract and considering the known efficacy of certain probiotics in the competitive niche in the GI tract, it seems logical to propose that probiotics may also be active in the oral cavity in maintaining oral health. However, the compositions of the bacterial communities inhabiting the mouth and those of the large intestine are quite different. Thus, it is not given that a particular strain with a proven intestinal effect may be the optimal strain in an oral environment.

FUTURE RESEARCH

The human microbiota and its function is complex and research needs to continue with the objective of better understanding microbe-microbe interactions as well as microbe-host interactions under both normal and pathological conditions. Future research needs to enumerate many of the dominant microbes present in the GI tract, and isolate and investigate the capacity of microbes to perform defined processes.

Some of the clinical trials included in this thesis might be regarded as limited. Large-scale well-controlled clinical trials with prolonged follow-up periods would further verify the efficacy of *L. reuteri* in e.g. chronic periodontal inflammation. Questions around how probiotics affect the formation of oral biofilms and how they affect the resident biota throughout life need to be answered. How is colonization by probiotics affected by the age of the individuals? These issues need to be addressed to understand the full benefits of probiotics in the oral cavity.

The remaining human breast milk samples from the global epidemiological study could be evaluated with molecular-based genomic methods (16S rRNA via RFLP), since the sequence of this gene is specific to a given bacterial species, thus enabling identification/composition of the various species present, and provide an improved knowledge of the LAB bacteria that are found in breast milk. Further studies are needed to provide a better understanding of the significance of *L. reuteri* and other lactic acid bacteria found in breast milk and their relevance to the health of the infant and the mother.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Under tusentals år har människor använt laktobaciller för att förbättra hållbarheten, smaken och näringsvärdet hos känsliga livsmedel. Buset av probiotika, framför allt i form av olika bakteriearter tillsatta i livsmedel, är idag av allt större intresse. Probiotika definieras som "levande mikroorganismer som när de intas i tillräcklig mängd ger hälsoeffekter hos individen". De bakterier som vanligen används som probiotika är olika stammar av laktobaciller och bifidobakterier, i dagligt tal kallade mjölksyrabakterier. *Lactobacillus reuteri* är en naturligt förekommande bakterie hos människan, men man hittar den inte hos alla individer. Man har genom åren visat att intag av specifika stammar av den hälsofrämjande arten *L. reuteri* har en positiv effekt på människors hälsa, att den på ett positivt sätt hjälper till att stärka och balansera olika funktioner i vår kropp, men man har ännu ingen klar bild över mekanismerna bakom dessa effekter.

Matsmältningskanalen är i sin helhet från mun till ändtarm koloniserad av olika mikroorganismer, huvudsakligen bakterier. Tjocktarmen är den del av människokroppen som har den största mängden och är ett extremt metaboliskt aktivt organ med stor betydelse för människans hälsa. När matsmältningskanalens bakterieflora är i obalans eller störs av stress, alkohol, antibiotika, onyttig mat, utlandsresor eller läkemedel kan sjukdomsframkallande bakterier få övertaget och orsaka olika typer av besvär. Probiotika kan återställa dessa komplexa mikrobiella ekosystem och förbättra matsmältningskanalens funktion genom att exempelvis konkurrera om näring och utrymme på bindningsställena på tarmcellerna, förbättra immunförsvaret och producera ämnen som hämmar andra mikroorganismer.

Ekosystemet i matsmältningskanalen är oerhört komplext och vi har ofullständig kunskap om de aktiviteter och den växelverkan som sker hos den medfödda mikrofloran. Denna avhandling syftar till att undersöka vilken roll *L. reuteri* har i dessa komplexa mikrobiella samhällen och få en bättre förståelse för sambanden mellan vår hälsa och förekomsten av *L. reuteri* i kroppen.

Resultaten från avhandlingen visar att *L. reuteri* förekommer naturligt i human bröstmjolk. Bakterien påvisades hos var sjunde ammande kvinna från geografiskt spridda länder. Bröstmjolk kan anses vara ett naturligt synbiotikum och resultaten från denna studie tyder på att *L. reuteri* är en av komponenterna i detta avseende. Intag av *L. reuteri* av gravida mammor och deras spädbarn resulterade i förekomst av *L. reuteri* i mammans bröstmjolk respektive i barnets avföring. Vidare reducerade *L. reuteri* gingival- samt plackindex hos patienter med måttlig eller svår gingivit, vilket tyder på minskad tandköttsinflammation. Bakteriell antagonism är en trolig orsak till minskad gingivit. Intag av *L. reuteri* resulterade i förekomst av *L. reuteri* i saliv, men ingen signifikant ändring visades i den undersökta supra- och subgingivala mikrofloran. Den signifikanta ökningen i plackindex i kontrollgruppen, utan motsvarande förändring i testgruppen, kan tyda på en probiotisk effekt av *L. reuteri* i denna population av friska individer.

STRESZCZENIE

Bakterie kwasu mlekowego tzw *Lactobacillus* były wykorzystywane przez człowieka od tysięcy do przechowywania żywności w celu zachowania jak największej wartości odżywczej i smakowej. Wykorzystywanie probiotyków, gdzie różnego rodzaju żywe mikroorganizmy są dodawane do żywności budzą coraz to większe zainteresowanie. Probiotykę definiuje się jako naukę o żywych organizmach, które po spożyciu w określonych ilościach wywierają korzystne efekty zdrowotne. Najczęściej wykorzystywanymi probiotykami to bakterie z rodzaju *Lactobacillus* oraz *Bifidobakterium* potocznie nazywane bakteriami mlekowymi.

Układ pokarmowy od ust do odbytu zawiera we wszystkich swoich częściach kolonie różnych mikroorganizmów głównie bakterii. Najbardziej zagęszczona ich populacja znajduje się w jelicie grubym, które jest organem ekstremalnie aktywnym metabolicznie i ma ogromne znaczenie dla zdrowia człowieka. W miarę, gdy flora bakteryjna układu pokarmowego traci równowagę lub zakłócana jest przez stres psychiczny, alkohol, antybiotyki, niezdrową żywność, zmianę sposobu żywienia np. podczas podróży zagranicznych lub środki farmaceutyczne, drobnoustroje patogenne mogą zaważać organizm i stać się powodem wielu dolegliwości. Probiotyka może odbudować funkcję kompleksowego ekosystemu mikroflory układu pokarmowego na przykład poprawiając układ odpornościowy, poprzez produkcję substancji hamujące wzrost szkodliwych mikroorganizmów oraz ograniczając możliwości kolonizacji tej niszy ekologicznej przez bakterie patogenne.

Ekosystem w układzie pokarmowym jest niesłychanie kompleksowy, a nasza wiedza o reakcjach i procesach we naturalnej mikroflorze jest jeszcze niepełna. Celem tej pracy jest zbadanie wpływu szczepu *Lactobacillus reuteri* na to kompleksowe środowisko mikrobakteryjne oraz lepsze zrozumienie ich efektów i znaczenia świata mikroflory dla zdrowia człowieka.

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