Improving the functional properties of *Lactobacillus reuteri*

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Cover: *Lactobacillus reuteri* DSM 17938
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Abstract

Lactobacillus reuteri is one of the most clinically studied probiotic bacteria. It has *e.g.* been shown to reduce the incidence and alleviate infantile colic and acute diarrhoea in children aged 3-60 months. The mechanisms of action behind the clinical effects are not fully understood, but two possibilities are inhibition of increased intestinal permeability (leaky gut) and alleviation of pain perception through inhibition of one of the key receptors (TRPV1) involved in visceral pain. The most studied strain of *L. reuteri* is DSM 17938, which has demonstrated good effects in clinical studies.

To study whether DSM 17938 and other strains could produce bioactive metabolites with a possible inhibitory effect against leaky gut syndrome, different varieties of barley flour were used as growth substrate. Growth and metabolic activity were analysed for different *L. reuteri*-barley flour combinations, through an overview of the metabolic profile and more targeted chemical analyses. Catabolites of tryptophan previously shown to be bioactive compounds (*e.g.* AhR-ligands) were detected. Supernatants from *L. reuteri*-barley flour combinations with high and low concentrations of these catabolites were evaluated for possible protective effect against increased permeability in an intestinal epithelial cell model. Only a minor protective effect was seen for a few supernatants and no correlations were found between content of AhR-ligands and effect on leakage.

In further attempts to improve the clinical effect of *L. reuteri*, experiments were performed to enhance certain functional properties through the use of experimental evolution. DSM 17938 has been shown to have flaws in certain properties that might contribute to the probiotic efficacy, *e.g*. freeze-thaw tolerance, bile tolerance and binding to intestinal mucus. Through experimental evolution, these properties were improved for DSM 17938 and, although genetic differences were only seen in two variants compared with the wild type, differences in protein expression were seen for all new variants.

Evaluation of the new variants in cell culture models showed that they had similar effects to wild-type DSM 17938 on inhibition of TRPV1, although *in vitro* tests using intestinal epithelial cells revealed that some of the variants had lost their inhibitory effect against increased permeability. Overall, however, the variants retained most of the properties seen *in vitro* for wild-type DSM 17938.

Keywords: Lactobacillus reuteri, barley, metabolomics, bile tolerance, mucus binding, experimental evolution, epithelial permeability, TRPV1, proteomics

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Förbättring av funktionella egenskaper hos *Lactobacillus reuteri*

Sammanfattning

Lactobacillus reuteri är en av de mest kliniskt studerade probiotiska bakterierna. Den har bland annat visats sig kunna minska frekvensen av och mildra kolik hos spädbarn och akut diarré för barn mellan 3-60 månader. Mekanismerna bakom de kliniska effekterna är inte helt förstådda men två av de föreslagna mekanismerna är inhibering av en ökad permeabiliteten i tarmen (läckande tarm) och lindring av smärtupplevelsen genom inhiberande effekt på en av de huvudsakliga receptorerna (TRPV1) involverade i visceral smärta. Den mest studerade stammen av *L. reuteri* är DSM 17938, som har visat sig ha bra effekt i en lång rad kliniska studier.

För att studera om DSM 17938 och andra stammar av *L. reuteri* kunde producera bioaktiva metaboliter med en möjlig inhiberande effekt mot läckande tarm, användes olika sorters kornmjöl som tillväxtsubstrat. Tillväxt och metabolisk aktivitet analyserades för olika kombinationer av reuteri och kornmjöl, genom en total översikt av metaboliska profiler och mer riktade kemiska analyser. Kataboliter av tryptofan som tidigare visat sig vara bioaktiva substanser (t.ex. AhR-ligander) kunde detekteras. Supernatanter från kombinationer av reuteri och kornmjöl med hög och låg koncentration av dessa kataboliter utvärderades för en möjlig skyddande effekt mot ökad permeabilitet i en cellmodel med tarmepitelceller. Bara en mindre skyddande effekt sågs för enstaka supernatanter och inga kopplingar hittades mellan innehåll av AhR-ligander och effekt mot läckage.

I ytterligare försök att öka den kliniska effekten av *L. reuteri*, gjordes försök att förbättra vissa funktionella egenskaper genom användning av experimentell evolution. DSM 17938 har visat sig ha brister i vissa egenskaper av möjlig betydelse för en probiotikans effekt, bland annat frys-tinatolerans, tolerans mot galla och bindning till mucus från tarmens. Genom experimentell evolution förbättrades dessa egenskaper för DSM 17938 och trots att genetiska skillnader bara hittades i två av de nya varianterna jämfört med vildtypen, sågs skillnader i proteinuttrycket för samtliga nya varianter. Utvärdering av de nya varianterna i cellmodeller visade att varianterna hade liknande effekt jämfört med vildtypen vid inhibering av TRPV1, dock hade vissa varianter tappat den inhiberande effekten mot en ökad permeabilitet. Överlag hade dock varianterna behållit de flesta av vildtypens egenskaper som testades *in vitro*.

Nyckelord: Lactobacillus reuteri, korn, metabolomik, tolerans mot gallsalter, bindning till mucus, experimentell evolution, epitelpermeabilitet, TRPV1, proteomik

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Dedication

To me, myself and Alice

You're going to find that many of the truths we cling to depend greatly on our own point of view

Obi-Wan Kenobi

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Anton Pallin*, Peter Agback, Hans Jonsson, Stefan Roos (2016). Evaluation of growth, metabolism and production of potentially bioactive compounds during fermentation of barley with *Lactobacillus reuteri*. *Food Microbiology* 57, 159-171.

II Anton Pallin, Jennifer Spinler, Hans Jonsson, Stefan Roos* (2018). Improving the ecological fitness of *Lactobacillus reuteri* DSM 17938 by experimental evolution (manuscript).

III Anton Pallin, M. Firoz Mian, Andrew M. Stanisz, John Bienenstock, Torbjörn Lundh, Hans Jonsson, Stefan Roos* (2018). *In vitro* evaluation of *Lactobacillus reuteri* DSM 17938 variants with improved stress tolerance and mucus binding capacity (manuscript).

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The contribution of Anton Pallin to the papers included in this thesis was as follows:

I Planned the studies with the co-authors, performed most laboratory work and had main responsibility for writing and revising the manuscript.

II Planned the studies with the co-authors, performed most laboratory work and had main responsibility for writing and revising the manuscript.

III Planned the studies with the co-authors, performed all laboratory work and had main responsibility for writing and revising the manuscript.

Abbreviations

AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
BSH	Bile salt hydrolase
cFDA	Carboxyfluorescein diacetate
CFU	Colony-forming units
ETEC	Enterotoxigenic *Escherichia coli*
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IBS	Irritable bowel disease
MLSA	Multi-locus sequence analysis
MRS	De Man, Rogosa and Sharpe
NMR	Nuclear magnetic resonance
OD	Optical density
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
QTOF-LCMS	Quadrupole Time of Flight Liquid Chromatography Mass Spectrometry
TEER	Transepithelial electrical resistance
TJ	Tight junction
TRPV1	Transient receptor potential vanilloid 1

1 Introduction

1.1 For a healthy intestine

It was long believed that the human body contained around 10 times or even 100 times as many bacterial cells as human cells (Savage, 1977). This claim is contradicted in a recent publication and the numbers are suggested to be closer to a 1:1 ratio, with a total mass of approximately 0.2 kg of bacteria in a person weighing 70 kg (Sender *et al.*, 2016). The microbiome of an adult human is suggested to contain hundreds to thousands of species, with the majority belonging to the phyla Bacteroidetes and Firmicutes (Huttenhower *et al.*, 2012; Rajilić-Stojanović *et al.*, 2007). Most of these bacteria (around 10^{13}) are found in the colon and contribute about half the weight of the colon contents (Sender *et al.*, 2016). The reason that the gastrointestinal tract can house such a massive number of bacterial cells is because of its sheer size. Measuring a total of 32 m^2, roughly the size of half a badminton court, this is one of the body's largest external surfaces (Helander & Fändriks, 2014). It has long been believed that the human gastrointestinal tract is colonised during and after birth (Mackie *et al.*, 1999), but this widely accepted hypothesis has been questioned during the past decade. Studies suggest that the foetus might not be sterile at all (Walker *et al.*, 2017; Jiménez *et al.*, 2008). However, this remains to be confirmed, since the majority of the findings have been of bacterial DNA, and not viable bacteria (Perez-Muñoz *et al.*, 2017). Even if the foetus is not completely sterile, the majority of colonisation happens after birth and the composition of the intestinal microbiota is believed to be set at an early age (Palmer *et al.*, 2007). The intestinal microbial community of an infant has low diversity, low numbers of bacteria (bacterial load) and low resilience, and usually consists of bacteria from the vagina, skin and breast milk of the mother (Dominguez-Bello *et al.*, 2010). Initially, the intestinal environment is oxidative, which allows facultative

anaerobic bacteria such as proteobacteria (*e.g. Escherichia coli*) and certain lactobacilli to colonise the intestines of infants (Sommer *et al.*, 2017). These bacteria are then responsible for their own replacement by decreasing the oxygen concentration, which in turn allows anaerobic bacteria such as those belonging to the phyla Bacteroidetes, Actinobacteria and Firmicutes to colonise (Sommer *et al.*, 2017). This results in a fluctuating microbiota during early life, but over the course of the first three years of life the microbial diversity increases and a more stable microbial community is established, and in turn a more resilient microbiota (Yatsunenko *et al.*, 2012; Koenig *et al.*, 2011). The microbiota is then relatively stable throughout the life time of healthy individuals (Rajilić-Stojanović *et al.*, 2013), although certain factors can influence the composition, including physiological state, drugs, disease, diet and stress (Sommer *et al.*, 2017; Lozupone *et al.*, 2012; Mitsuoka, 1992). Having a stable intestinal microbiota with a normal composition is considered important to avoid disease, and has been studied as far back as the 1950s. For example, Bohnhoff *et al.* (1954, *cit.* Schrezenmeir & de Vrese, 2001) showed that mice treated with antibiotics become more susceptible to *Salmonella* infections(Schrezenmeir & de Vrese, 2001). Similar observations have been made for guinea pigs following oral administration of a specific antibiotic, which left the animals more susceptible to *Vibrio cholerae* (Freter, 1955). In older humans, changes in microbiota composition and, especially, loss of diversity are associated with increased frailty and reduced cognitive performance (O'Toole & Jeffery, 2015). This loss of microbial diversity and the health decline could be correlated with a less diverse diet (Claesson *et al.*, 2012).

A stable microbiome does not just contribute to defence against pathogenic microorganisms, since changes or disruptions in the composition of the microbiome are also correlated with several different diseases and syndromes. Reports of observed changes in microbiome composition in different diseases are continually emerging. For example, differences in microbiome composition compared with healthy subjects have been observed in people with obesity, circulatory disease, inflammatory bowel disease and autism (Tomova *et al.*, 2015; Lepage *et al.*, 2011; Furet *et al.*, 2010; Qin *et al.*, 2010; Turnbaugh *et al.*, 2009; Finegold, 2008; Holmes *et al.*, 2008; Marchesi *et al.*, 2007; Turnbaugh *et al.*, 2006). Moreover, an influence of the intestinal microbiome on drug metabolism and toxicity, dietary calorific bioavailability and recovery after surgery has been reported (Kinross *et al.*, 2010; Clayton *et al.*, 2006; Hooper & Gordon, 2001). A physiological effect resulting from disruption of the stable microbiome, or dysbios, is a condition often referred to as 'leaky gut' syndrome, which has been linked to several different diseases (Frazier *et al.*, 2011; Sartor, 2008).

1.1.1 Leaky gut

Leaky gut syndrome, or 'increased intestinal permeability', describes an inability of the intestine to prevent harmful substances or microorganisms from entering the internal environment of the body (Bjarnason *et al.*, 1995). The intestine consists of a number of different barriers to prevent this from happening (Atuma *et al.*, 2001). These barriers can roughly be divided into three types: immunological, biochemical and physical elements. The immunological elements include antimicrobial peptides (defensins), secretory immunoglobulins and cellular immunity (dendritic cells, macrophages, intraepithelial lymphocytes, T regulatory cells, lymphocytes and plasma cells). Biochemical elements are bile salts, gastric acid, lysozymes *etc*. Physical elements, the main focus in this thesis, include the commensal microbiota, the mucus layer and the intestinal epithelium (see Figure 1). The commensal microbiota compete with invasive microorganisms by limiting nutrients and attachment spots for possible pathogens and/or by the production of antimicrobial substances (see section 1.1 of this thesis). The mucus layer consists of one or more layers of highly glycosylated hydrated gels formed by the mucin (large glycoproteins) produced by goblet cells, which are simple columnar epithelial cells scattered around the epithelial lining of *e.g*. the intestinal tract (Atuma *et al.*, 2001). The tight stacking of these glycoproteins forms a compact inner layer closest to the epithelial layer that is considered to contain far smaller numbers of bacteria (Dicksved *et al.*, 2012; Johansson *et al.*, 2008; Swidsinski *et al.*, 2007). The outer mucus layer is looser because of proteolytic dispersion, and hence contains more intestinal microorganisms (Atuma *et al.*, 2001). The epithelial layer in turn consists of two barriers (Figure 1). The cell membrane consists of lipids, which prevent water-soluble substances from passing through (Hollander, 1999). Between each epithelial cell there is a second barrier, where the gap between cells, called the paracellular space, contain protein complexes that control what can/cannot enter this space. These proteins are called tight junction (TJ) proteins. When open, they can allow fluids, nutrients and even microorganisms to cross from the lumen into the lamina propria of the intestine (Madara & Pappenheimer, 1987). Leaky gut occurs when the function of these TJ proteins is disturbed, *e.g*. during certain diseases such as inflammatory bowel disease (Lee, 2015; Krug *et al.*, 2014).

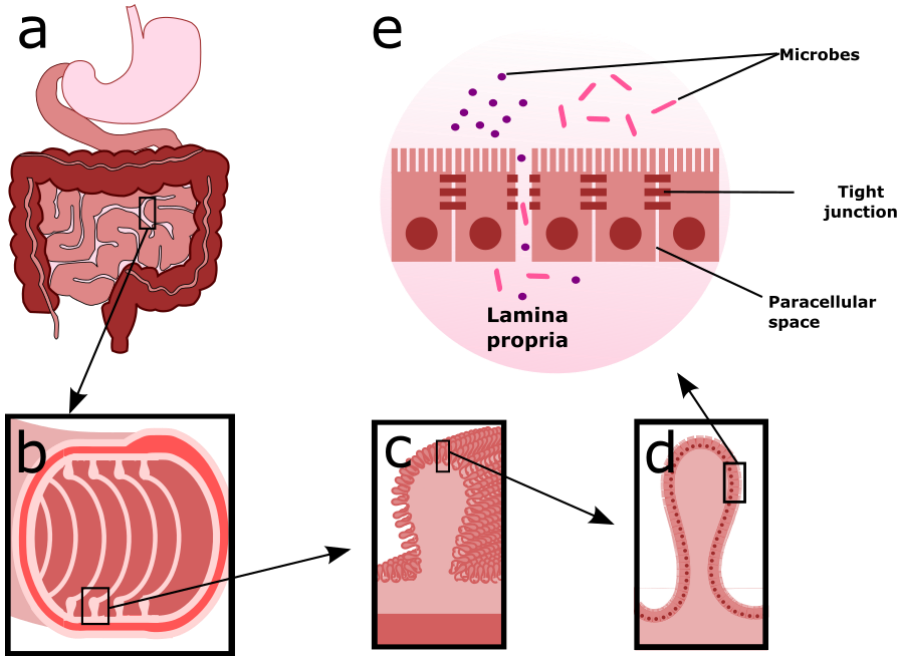

Figure 1. Simplified overview of the structure of the small intestine, showing a) the gastrointestinal tract, b) intersection of the small intestine with its folds, c, d) villi and e) individual epithelial cells with microvilli. The epithelial layer comprises a single cell layer where the paracellular space is sealed by tight junction (TJ) proteins, the function of which is to limit leakage of unwanted substances or microorganisms. A leaky gut, or increased intestinal permeability, can be caused by disturbed function of TJ proteins, leading to microbes entering the lamina propria.

Studies in the 1980s examined the correlations between Crohn's disease and coeliac disease, and leaky gut. These studies found that subjects suffering from Crohn's disease had increased intestinal permeability of lactulose, which is a non-absorbable disaccharide suggested to be taken up only via *e.g.* tight junctions (Ukabam *et al.*, 1983; Pearson *et al.*, 1982). Similar results were obtained for subjects with coeliac disease, with higher permeability of similar-sized molecules (Ukabam & Cooper, 1984; Bjarnason *et al.*, 1983). The effect is reversed by excluding gluten from the diet of the latter subjects, leading to normal intestinal permeability (Ukabam & Cooper, 1984). An increase in intestinal permeability has also been reported in subjects suffering from other gastrointestinal disorders, *e.g.* patients suffering from diarrhoea-predominant irritable bowel syndrome (IBS) (Camilleri *et al.*, 2012). These people have higher ratios of lactulose/mannitol in urine samples than healthy controls, suggesting the same leakage of molecules through the tight junctions as in patients with coeliac and Crohn's disease. This has been shown in several studies on people with IBS, and correlations between increased permeability and

abdominal pain have been reported in some of these studies (Zhou *et al.*, 2009; Dunlop *et al.*, 2006; Spiller *et al.*, 2000). However, those studies also suggest connections between other disorders and diseases, including food allergies, asthma and some allergic skin conditions, and leaky gut.

Understanding these connections and knowing how to measure intestinal permeability could be paramount in the development of preventative measures and early detection of these diseases and disorders. Protection against leaky gut could prevent people suffering from painful and bothersome disorders. Probiotics have been suggested as a possible treatment and the role of probiotics in decreasing intestinal permeability has been evaluated in a number of studies. For example, giving probiotic supplements (*Lactobacillus rhamnosus* 19070-2 and *L. reuteri* DSM 12246) to children with severe to moderate dermatitis has been shown to decrease the gastrointestinal symptoms (diarrhoea and abdominal pain) and intestinal permeability (lactulose/mannitol test) compared with a placebo (Rosenfeldt *et al.*, 2004). Treating children suffering from mild to moderately active Crohn's disease with *L. rhamnosus* GG is reported to result in lower disease scores and lower intestinal permeability (cellobiose/mannitol ratio) (Gupta *et al.*, 2000). A protective effect against increased intestinal permeability, often seen in pre-term infants, has been observed following ingestion of pre-term formula supplemented with *Bifidobacterium animalis* subsp. *lactis* (Stratiki *et al.*, 2007). Intestinal epithelial cell models are available for studying how probiotics prevent leaky gut syndrome. These include *e.g.* IPEC-J2 (primary porcine cell line) and Caco-2 (human carcinoma cell line), two cell lines that can form polarised monolayers which express tight junction complexes (Kandil *et al.*, 1995; Grasset *et al.*, 1984). Probiotics such as *L. reuteri* have been shown to have a protective effect against increased intestinal permeability in IPEC-J2 (Karimi *et al.*, 2018; Liu *et al.*, 2015). These studies show a protective effect of *L. reuteri* on the permeability-disrupting effects of enterotoxigenic *Escherichia coli* (ETEC), with a reduction in transepithelial resistance, leakage of a fluorescent molecule (FITC-dextran, 4kDa) and monolayer damage (tight junction openings) caused by ETEC after pre-treatment with *L. reuteri.*

1.2 Probiotics

The field of probiotics is considered to have started in the beginning of the twentieth century, with Elie Metchnikoff. He postulated that alteration of the intestinal microbial balance could have health implications. Those observations led to his hypothesis that consumption of yoghurt containing host-friendly bacteria could decrease toxin-producing bacteria in the intestine and increase the

longevity of the host (Schrezenmeir & de Vrese, 2001). Metchnikoff also introduced the concept of probiotic bacteria, by combining the Latin word "pro" meaning "*for*" and the Greek word "*bios*" meaning "*life*". The actual term probiotics was coined in 1953 by a scientist named Werner Kollath, referring to active substances promoting health (Kollath, 1953). The term was later refined to describe growth-promoting factors produced by microorganisms (Lilly & Stillwell, 1965). However, the definition mostly used today is: "Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit to the host". This definition was first established by the World Health Organisation (WHO) and the Food and Agriculture Organization of the United Nations (FAO) in 2001.

Certain criteria are usually considered important when developing a probiotic product (Dunne *et al.*, 2001). First, the microorganism should be host-specific, meaning that it should come from the same species as the intended target group, *i.e.* human microorganisms for human probiotic products and canine microorganisms for dogs. Second, the probiotic should be able to survive passage through the gastrointestinal tract to reach the intended area of action. This means that it should be able to survive the pancreatic enzymes, acids and bile challenges that it will encounter from the mouth down to the small intestine or large intestine. Third, in order to establish (at least temporarily) in the intestinal microbial community, it is important for microorganisms to be able to adhere to the different mucosal layers of the intestinal lining. This could also be an important attribute for immunomodulation, pathogen exclusion and enhanced healing of damaged mucosa. Fourth, for pathogen exclusion, but also for persistence, production of anti-microbial substances is a desirable characteristic for probiotic candidates. Fifth, other more technical properties can be important, especially for production on an industrial scale. These include genetic stability, lyophilisation tolerance and stability over time (shelf life), good and fast growth, and oxygen tolerance. Last but not least, the probiotic candidate obviously needs to have a documented beneficial health effect and no or minor side-effects.

1.2.1 Health effects of probiotics

The suggested health effects of probiotics are many, including positive effects on gut health, oral health, reduction of allergic reactions, boosting of the immune system and effects against certain psychological disorders. These suggested health effects can be divided into well-documented outcomes with good evidence according to meta-analyses, and health effects with low evidence where no meta-analyses have been performed or where meta-analyses have

failed to find enough evidence for the suggested effect. Examples of health effects from probiotic intake supported by good evidence include:

- Reduction in colic symptoms in breastfed infants (Schreck Bird *et al.*, 2017; Sung *et al.*, 2017)
- Prevention of severe necrotising enterocolitis, late-onset sepsis and all-cause mortality in very low birth weight infants (Athalye-Jape *et al.*, 2018; Dermyshi *et al.*, 2017)
- Enhancing efficacy of *Helicobacter pylori* eradication treatment and decreasing side-effects (Wang *et al.*, 2017; Tong *et al.*, 2007)
- Prevention of antibiotic-associated diarrhoea (Blaabjerg *et al.*, 2017; Shen *et al.*, 2017)
- Increased stool frequency and decreased intestinal transit time (Huang & Hu, 2017; Miller *et al.*, 2017)
- Relieving symptoms of irritable bowel syndrome (Yuan *et al.*, 2017; Ortiz-Lucas *et al.*, 2013)

Examples of health effects for which further evidence is needed include:

- Reduction in allergic symptoms or prevalence of allergy in infants (Cuello-Garcia *et al.*, 2015; Zajac *et al.*, 2015)
- Treatment of subclinical psychological symptoms, including depression and anxiety (Huang *et al.*, 2017; McKean *et al.*, 2017; Huang *et al.*, 2016)
- Managing caries and periodontitis (Gruner *et al.*, 2016; Martin-Cabezas *et al.*, 2016)

An review article published in 2014 lists at least 25 different species of bacteria and yeast with demonstrated probiotic effects (Fijan, 2014). These species belong to many different genera, including *Bacillus*, *Enterococcus*, *Escherichia*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Saccharomyces* and *Streptococcus*. However, the most frequently used and most well-studied species belong to the genera *Lactobacillus* and *Bifidobacterium* (Fijan, 2014). Among the genus *Lactobacillus*, one of the most studied species is *Lactobacillus reuteri.*

1.2.2 Lactobacillus reuteri

Lactobacillus reuteri was first isolated in 1962 by microbiologist Gerhard Reuter. He noticed differences between *L. reuteri* and *Lactobacillus fermentum*, but still classified *L. reuteri* as *L. fermentum* biotype II. It was not until 1980 that Otto Kandler and his group described it as a separate species, and named it after

Reuter who first isolated it (Kandler *et al.*, 1980). *Lactobacillus reuteri* is a gastrointestinal tract-associated bacteria and can be found in a wide variety of animal hosts, including poultry, pigs, dogs, wolves, rodents *etc*. (Walter *et al.*, 2011; Hammes & Hertel, 2006). Before the modern Westernised lifestyle and diet had its detrimental effects on the microbiome of humans, *L. reuteri* is believed to have been present in the gastrointestinal tract of the majority of human populations. Back in the 1960s and 1970s, it was regularly detected in the intestine and was considered to be one of the dominant *Lactobacillus* species (Walter *et al.*, 2011). By 1993, however, a study reported that *L. reuteri* was only detected in 4% of study subjects (Molin *et al.*, 1993). A study from 2015, comparing people from non-industrialised areas in Papua New Guinea and people from the United States, revealed that the microbial diversity was significantly higher in the former group. One of the species that was most affected was *L. reuteri*, which was present in all the subjects from Papua New Guinea, but was mostly absent in people from the United States (Martínez *et al.*, 2015).

The only environment outside the body of humans and other warm-blooded animals where *L. reuteri* has been isolated is in high-temperature sourdoughs. However, these isolates are most likely to be faecal contaminants of rodent origin (Su *et al.*, 2012). Adapting to different conditions by functional and sometimes host-specific properties has made it possible for *L. reuteri* to exist in many different animals. The differences in phenotypes seen between strains include an ability to adhere to mucus or epithelial cells (sometimes host-specific binding) and the production of certain enzymes (such as urease) or anti-microbial substances (*e.g*. reuterin) (Mora & Arioli, 2014; Walter *et al.*, 2011; MacKenzie *et al.*, 2010). *Lactobacillus reuteri* belongs to the group of host-adapted lactobacilli, according to a recent review article presenting an updated core phylogenomic tree (Duar *et al.*, 2017). That article divides lactobacilli into three groups (free-living, host-adapted and nomadic bacteria) based on the isolation source, frequency of isolation, metabolic capabilities, growth temperature and ability to withstand environmental stressors present in given habitats. The host-adapted lactobacilli usually have a smaller genome size, have more specialised properties and lack the metabolic flexibility seen in nomadic lactobacilli (Duar *et al.*, 2017).

Like most other lactobacilli, *L. reuteri* has high requirements as regards nutrient supply and needs easily fermentable sugar, amino acids, vitamins and nucleotides in order to grow. If all those requirements are fulfilled, it has a duplication time of less than one hour (Gerez *et al.*, 2008). Carbohydrates are fermented using the phosphoketolase pathway to form lactic acid, acetic acid, ethanol and carbon dioxide. Since this pathway has a low energetic yield, *L.*

reuteri makes use of external electron acceptors, including fructose, glycerol, pyruvate, citrate, nitrate and oxygen, to improve this (Årsköld *et al.*, 2008). Despite being a demanding bacterial species, it has been shown to be resilient to environments with both low pH and the presence of bile salts (Whitehead *et al.*, 2008; Jacobsen *et al.*, 1999). As mentioned previously, these could be important characteristics for a probiotic strain. Some strains of *L. reuteri* also have the ability to produce antimicrobial substances. For example, lactic and acetic acid, which *L. reuteri*, and other lactic acid bacteria, produce during degradation of carbohydrates, have been shown to inhibit many microbes, especially Gram-negative bacteria (In *et al.*, 2013; Alakomi *et al.*, 2000). Another anti-microbial substance produced by certain strains of *L. reuteri*, including strains of dog, human, pig and poultry origin (Walter *et al.*, 2011), is 3-hydroxypropionaldehyde (3-HPA) (reuterin) (Spinler *et al.*, 2014). During utilisation of glycerol, these strains can produce this low molecular weight, water-soluble, non-proteinaceous and neutral end-product (Spinler *et al.*, 2008). Reuterin is formed during anaerobic growth by dehydration of glycerol, and *L. reuteri* can then reduce reuterin to 1,3-propanediol, to regenerate NAD+ from NADH to enhance its growth (Schaefer *et al.*, 2010). It can be accumulated by *L. reuteri* and excreted under certain conditions, *e.g*. the presence of other bacteria such as *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Staphylococcus*, *Clostridium* and *Pseudomonas* has been shown to stimulate *L. reuteri* to convert glycerol into reuterin (Schaefer *et al.*, 2010; Chung *et al.*, 1989). The production of reuterin possibly gives *L. reuteri* a competitive advantage in the gastrointestinal tract (Cleusix *et al.*, 2007). It has also been shown to inhibit growth of Gram-negative and Gram-positive bacteria, fungi, protozoa and even viruses (Walter *et al.*, 2011; Schaefer *et al.*, 2010; Jacobsen *et al.*, 1999; Axelsson *et al.*, 1989).

In addition to reuterin, the production of yet another antimicrobial substance, named reutericyclin, has been observed in some strains, so far exclusively in strains of sourdough origin (Zheng *et al.*, 2015). It is also a low molecular weight substance, but is a tetramic acid, and does not seem to affect as many different microorganisms as reuterin. Against Gram-positive bacteria it has been shown to be both bacteriostatic and bactericidal, but against Gram-negative bacteria it is ineffective due to the barrier properties of the outer membrane (Gänzle *et al.*, 2000).

Lactobacillus reuteri is one of the most clinically studied probiotic species, with many studies showing probiotic effects of some strains. Most of the studies showing strong clinically proven effects are on children and/or infants. Strain DSM 17938 has been shown to work in both therapeutic and preventive settings against acute diarrhoea in children between the ages of 3 and 60 months

(Urbańska *et al.*, 2016; Gutierrez-Castrellon *et al.*, 2014; Weizman *et al.*, 2005). Supplementation of the diet with strain DSM 17938 can also alleviate gastrointestinal tract symptoms (constipation and regurgitation) and improve colicky symptoms (reduce crying time) in breastfed infants (Sung *et al.*, 2017; Indrio *et al.*, 2014; Savino *et al.*, 2010). The risk of developing eczema can also be lowered by treating infants with different strains belonging to *Bifidobacterium* and *Lactobacillus*, one of the species being *L. reuteri* (Zuccotti *et al.*, 2015). Children with ulcerative colitis might also benefit from taking *L. reuteri*, although further studies are needed before an effect can be claimed (Oliva *et al.*, 2012). While most of the beneficial effects are seen in infants and young children, positive health effects have also been shown in adults. For example, in patients suffering from *Helicobacter pylori* infections, *L. reuteri* has been shown to reduce the overall occurrence of dyspeptic symptoms, abnormal defecation and flatulence compared with people receiving a placebo (Francavilla *et al.*, 2014; Francavilla *et al.*, 2008). Several studies have also examined the effect of *L. reuteri* on visceral pain. Studies performed using an array of cell models and mice models, as well as clinical studies on children, have all shown the potential of *L. reuteri* to inhibit visceral pain (Weizman *et al.*, 2016; Perez-Burgos *et al.*, 2015; Kamiya *et al.*, 2006).

Besides having an effect on a number of symptoms of different diseases and syndromes, a rodent *L. reuteri* strain (R2LC) has been shown to reduce transport of live bacteria from the intestinal lumen to the bloodstream (Wang *et al.*, 1995). This transport pathway, known as bacterial translocation, is a strategy used by pathogenic bacteria, but also by members of the commensal intestinal microbiota, when the host is left more exposed than usual, *e.g.* during stressful conditions such as starvation, trauma *etc*. (Balzan *et al.*, 2007; Berg, 1995). *Lactobacillus reuteri* has also been shown to have a positive effect on maintaining intestinal mucosal integrity (Adawi *et al.*, 1997). In addition, an effect on gut motility has been suggested as a possible mode of action for *L. reuteri*, *e.g.* strain DSM 17938 has been shown to lower jejunal velocity and increase colon velocity in studies using an *ex vivo* model (Wu *et al.*, 2013). This could explain the positive effect of DSM 17938 against chronic constipation seen in a study on children (Coccorullo *et al.*, 2010).

1.3 Probiotic characteristics

Previously, it was believed that a bacterial strain should fulfil certain criteria to be considered a good probiotic candidate. However, even if this is a good first step in screening strains, in the end the only effect that matters is that achieved in a clinical setting. This section provides a more detailed picture of the different

stresses that bacteria need to overcome and how they have evolved to handle different kinds of challenges. It also addresses the topic of production of bioactive compounds more extensively.

1.3.1 Stress tolerance

Lyophilisation tolerance/survival ability

Lyophilisation, or freeze-drying, is an important technique for the probiotic industry, as it permits longer storage of the bacteria with a slower decrease in viable counts, which is important since a high number of viable bacteria could affect the efficacy of the probiotic product. The process of lyophilisation involves freezing below the glass transition temperature of the formulation, followed by drying by sublimation under high vacuum (Jennings, 1999). Drying consists of two phases. In the first phase, called primary drying, unbound water is removed, while in the second phase the bound water is removed (Jennings, 1999). Removal of cellular water is needed to preserve the bacteria and lyophilisation is considered a gentle method for achieving this (Meng *et al.*, 2008). However, this process can have a detrimental effect on the bacteria if carried out improperly, resulting in damage to the cell wall and membrane constituents and leading to cell death (Carvalho *et al.*, 2004). To avoid damage to the cells, it is important to have a good cryoprotectant and lyoprotectant in the drying medium. Commonly used cryoprotectants and lyoprotectants include *e.g.* skim milk powder, whey protein, trehalose, glycerol, glucose, sucrose, lactose and polymers such as dextran (Meng *et al.*, 2008). On addition to the formulation, these substances form a protective matrix around the cells, which protects them from different kinds of harmful stresses during the process of freezing and drying (Wessman *et al.*, 2013). Addition of fermentable sugars to the growth medium could also have protective effects on the bacteria, as converting these sugars into *e.g*. mannitol, exopolysaccharides *etc*. could improve the viability during drying (Santivarangkna *et al.*, 2008). Supplementation with non-fermentable sugars could also help the cells during the drying phase (Santivarangkna *et al.*, 2008). These sugars exert a hyperosmotic stress on the bacterial cells during cultivation and, by accumulation of compatible solutes, the bacteria could be better equipped to resist the osmotic stress during the drying process. These accumulated solutes could better help to keep proteins and membranes hydrated (Santivarangkna *et al.*, 2008).

Studies examining the effect of different cryoprotectants on the survival ability of *L. reuteri* CICC6226 after lyophilisation have found that trehalose and

skim milk are the best, closely followed by sucrose (Li *et al.*, 2011). A study performed on *L. reuteri* DPC16 also found sucrose to be one of the better cryoprotectants (Chen *et al.*, 2011). Tolerance to repeated cycles of freezing and thawing is suggested to correlate with an increase in stability and/or survival ability of *L. reuteri* after lyophilisation (Garcia et al., unpublished). The most used method for evaluating viable counts after lyophilisation is quantification of colony-forming units (CFU) by plate methods. Because of its drawback with long incubation times of plates of 24-72 hours or more, other methods are being developed for more rapid testing of viability. These methods include *e.g.* fluorescent techniques like flow cytometry to detect live, dead and damaged cells by staining with different fluorescent probes (Rault *et al.*, 2007), isothermal calorimetry which measures the heat produced by metabolising bacteria (Garcia *et al.*, 2017), and real-time quantitative polymerase chain reaction (qPCR) in combination with DNA-binding dyes to detect both live cells and total cell counts (Shao *et al.*, 2016).

Tolerance to low pH

The first challenge after ingestion of a probiotic product is the transport through the stomach. Located in the corpus of the stomach are highly specialised cells called parietal cells (Martinsen *et al.*, 2005). These cells secrete hydrochloric acid (HCl), which causes an increase in the concentration of H^+ ions and a drastic drop in pH. The pH in the human stomach, as in that of many other omnivores, is around pH 3, but the pH mainly depends on how much protein is consumed. Carnivores usually have a lower pH, since the secretion of pepsinogen and the activity of proteases require a low pH (Beasley *et al.*, 2015). Another function of the gastric juice is inactivation of pathogenic microorganisms. Studies have shown a bactericidal effect of pH lower than 4, with the potential to kill bacteria within 15 minutes (C. *et al.*, 2005). However, more recent studies have speculated that low stomach pH may be a double-edged sword, by also damaging the chances of re-colonisation by beneficial microbes that might need to be re-introduced (Beasley *et al.*, 2015). Bacteria are differently affected by low pH, *e.g.* Gram-negative bacteria are believed to have a lower resistance because of the inability of their outer membrane to act as a barrier against proton movement (Lund *et al.*, 2014). Gram-negative and Gram-positive bacteria have been shown to use different mechanisms for surviving exposure to low pH. Species belonging to the genus *Lactobacillus*, like many other organisms, use a sophisticated molecular machinery located in the plasma membrane called F_1F_0-ATPase (van de Guchte *et al.*, 2002). Protons can either be used to synthesise ATP or be expulsed out of the cells using the energy provided during ATP

hydrolysis (van de Guchte *et al.*, 2002). In lactic acid bacteria it is mainly the latter that is used to maintain the ΔpH (Nannen & Hutkins, 1991).

Bile acid tolerance

After surviving the acidic environment of the stomach, the next set of hurdles the bacteria need to overcome are the membrane-dissolving bile salts. These are formed in the hepatocytes of the liver from cholesterol by a multi-enzyme process (Hofmann, 1999). The insoluble, uncharged cholesterol is converted to a bile acid molecule, which when ionised becomes an amphipathic, membrane-dissolving and water-soluble detergent. When the bile salts have been synthesised from cholesterol and before they are secreted, they are conjugated with either glycine or taurine. This changes them from being weakly acidic to strongly acidic (Hofmann, 1999). Bile acids promote dietary lipid absorption in the small intestine. By solubilising dietary lipids and their digestion products in the form of mixed micelles, they can diffuse through the unstirred layer at an accelerated pace. If they are not in micellar form, the fat-soluble vitamins such as vitamin A, D, E and K are not absorbed (Hofmann, 1999). Another purpose of bile salt synthesis is elimination of cholesterol (Dikkers & Tietge, 2010). The cholesterol can be eliminated via the faecal route, by converting some of it to bile acids and then through micellar solubilisation of cholesterol in bile, enable cholesterol to move from the hepatocyte to the intestinal lumen.

Since bile salts are membrane-dissolving, they are also antimicrobial (Begley *et al.*, 2006). However, certain microbial species have the ability to hydrolyse bile salts entering the intestinal lumen, a process which mainly takes place in the anaerobic conditions of the colon (Begley *et al.*, 2006). Cytoplasmic bile salt hydrolases (BSH), mainly present in Gram-positive strains, can convert bile salts by deconjugation (Begley *et al.*, 2006). They hydrolyse the amide bond and liberate the glycine/taurine moiety from the steroid core, resulting in unconjugated or deconjugated bile acids. Bile salt hydrolases are present in species belonging to *e.g.* the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium* and *Bacteroides* (Begley *et al.*, 2006; Tanaka *et al.*, 1999). Their main purpose in bacteria having this activity is believed to be for bile detoxification, which allows them to be more persistent in the gastrointestinal tract (Dussurget *et al.*, 2002; De Smet *et al.*, 1995). However, BSH may also play a nutritional role, and can lead to membrane alterations by facilitating incorporation of cholesterol or bile into the membrane (Begley *et al.*, 2006; Taranto *et al.*, 1997; Tannock *et al.*, 1989), which could make the bacteria more resistant to bile, intestinal defensins, lysozymes *etc*.

1.3.2 Mucus binding

After surviving passage through the stomach and the first part of the small intestine, there is a new challenge for the probiotic bacteria. In order for a bacterium to persist in the intestine long enough to exert an effect, it must be able to bind to the mucosal lining of the intestinal cell layer. As mentioned (section 1.1.1 of this thesis), the intestinal layer consists of one or several layers of glycoproteins, forming a continuous gel matrix that is bound to the intestinal epithelia. The main function of this barrier is to protect the host from harmful antigens and microorganisms entering through the epithelial layer, but also to promote the transport of food and waste downwards through the gastrointestinal tract. The thickness of the mucus layer in the human intestinal tract varies from 30 to 300 µm, being thinnest in the small intestine and progressively increasing down the intestinal tract to be thickest in the rectum (Matsuo *et al.*, 1997).

As mentioned (section 1.1.1), the glycoproteins or mucins are produced by specialised cells in the epithelial layer called goblet cells. When the mucins produced by these unicellular cells polymerise, they form a matrix that gives protection from pathogens, enzymes, toxins, dehydration and abrasion (Van Tassell & Miller, 2011). Mucins are constantly being produced, since the mucus layer is subjected to erosion by luminal particular matter and by intestinal peristalsis. The secretion of mucin can be increased due to a number of different factors, including luminal acid, neuro- and inflammatory mediators, hormone-like lipids called prostaglandins (Akiba *et al.*, 2000) and by the presence of bacteria through the release of so-called effectors (Sicard *et al.*, 2017). The mucins can either be translocated to the membrane surface or secreted into the mucus matrix. The ability of lactobacilli to adhere to mucus varies greatly among species and strains (Rinkinen *et al.*, 2003; Kirjavainen *et al.*, 1998). The binding to the mucus layer can involve *e.g.* large surface proteins with highly repetitive structures, though the mechanism is yet to be fully understood. It has been suggested that carbohydrate-protein interactions are a possible mechanism (Roos & Jonsson, 2002). Studies have managed to describe proteins with mucus-binding motifs suggested to be conserved in numerous *Lactobacillus* species (Van Tassell & Miller, 2011; Antikainen *et al.*, 2009). Examples of such proteins produced by *L. reuteri*, are Mub (Roos & Jonsson, 2002) and CmbA (Jensen *et al.*, 2014). The adhesive properties of these proteins are considered to be due to the repeated functional domains within the proteins (Roos & Jonsson, 2002). Mub and homologs of these proteins are mainly found in lactic acid bacteria and lactobacilli isolated from the intestine of humans and other mammals (Van Tassell & Miller, 2011).

1.3.3 Production of bioactive compounds

Bioactive compounds are health-promoting substances which are naturally occurring parts of the diet (Kris-Etherton *et al.*, 2002). They can be either non-essential or essential compounds, but they need to have an established effect on health. Bioactive compounds can be isolated nutrients or part of dietary supplements, genetically engineered "designer" foods, herbal products or processed foods (Biesalski *et al.*, 2009). Although present in many different food ingredients, they are not always available for the host. For example, phenolic acids found in cereals are mainly bound to arabinoxylans through ester bonds, which cannot be hydrolysed by the digestive enzymes found in the human intestine (Saura-Calixto, 2010). However, some of these phenolic compounds can be released by certain intestinal microorganisms and made accessible to the host. For example, during fermentation of barley and oat by species of lactobacilli, including *L. reuteri*, an increase has been seen in levels of bioavailability for certain phenolic acids, *e.g.* caffeic, coumaric, ferulic and sinapic acid (Hole *et al.*, 2012). Fermentation of cowpea flour by *Lactobacillus plantarum* has been shown to increase the concentrations of phenolic compounds and also the antioxidant activity (Dueñas *et al.*, 2005). The concentrations of a major inhibitory neurotransmitter in the mammalian central nervous system, gamma-aminobutyric acid (GABA), can also be increased by fermentation of different kinds of cereals and sub-cereals by lactobacilli (Stromeck *et al.*, 2011; Coda *et al.*, 2010). Production of short-chain fatty acids, which are associated with reduced risk of diseases, *e.g.* cancer, cardiovascular disease and inflammatory bowel diseases, has also been shown to occur in probiotic bacteria (Floch & Hong-Curtiss, 2002; Jenkins *et al.*, 1999).

Lactobacillus reuteri is known to be involved in the production or release of a number of different bioactive compounds. Cobalamin (vitamin B_{12}), which is essential for humans, is produced by *L. reuteri* since it is needed as a co-factor for the enzyme converting glycerol into the anti-microbial substance reuterin (Sriramulu *et al.*, 2008; Taranto *et al.*, 2003). *Lactobacillus reuteri* has also been shown to play an important part in the metabolism of tryptophan into highly bioactive derivatives, including aryl hydrocarbon receptor (AhR) ligands, produced in low carbohydrate, high tryptophan containing medium fermented by *L. reuteri* (Zelante *et al.*, 2013). When interacting with the receptors, these ligands promote production of IL-22, which in turn mediates innate antifungal resistance and also mucosal protection against inflammation in a mouse model (Zelante *et al.*, 2013). Studies have also shown that faeces from humans with inflammatory bowel disease (IBD) transferred to mice give lower activation of AhR than faeces from healthy humans (Lamas *et al.*, 2016). In a mouse model, *L. reuteri* has also been found to induce a specific class of intestinal T-cells,

CD4+CD8αα+ double-positive intraepithelial lymphocytes (DP IELs), which are believed to be involved in a number of different immune responses, again possibly through production of specific derivatives of tryptophan (Cervantes-Barragan *et al.*, 2017).

1.4 Aims of the thesis

The overall aim of this thesis was to improve the functional properties of *Lactobacillus reuteri.* In Paper I, *L. reuteri* was cultivated in different types of cereal-based substrates, in order to study production of potential bioactive compounds. In Papers II and III, the focus was on improving specific functional properties connected to improved survival ability and/or increased retention time of the bacteria in the intestine, hence improving probiotic-host cell interactions.

Specific objectives of the work were to:

- Isolate and characterise new strains of *L. reuteri* from cereals (Paper I)
- Study the growth and general metabolism of isolated strains compared with commercial probiotic strains in barley fermentations (Paper I)
- Study production of bioactive compounds, with particular focus on tryptophan catabolites, during barley fermentation (section 3.2 of this thesis)
- Study the effect of fermented barley slurries on intestinal permeability in a small intestine epithelial cell model (section 3.2 of this thesis)
- Improve the freeze-thaw tolerance, bile tolerance and mucus binding capacity of *L. reuteri* DSM 17938 through experimental evolution (Paper II)
- Determine the genetic and proteomic changes linked to the changes in phenotypes of DSM 17938 (Paper II)
- Evaluate the stability of the improved functional properties (Paper II) and evaluate other possible changes connected to the changed functional properties (Paper III)
- Study and compare the effect of improved variants in different cell models (Paper III)

2 Materials and methods

The experiments reported in Paper I included isolation of new strains of *L. reuteri* from cereals, characterisation of said strains and evaluation of growth and metabolism of a selected few of the strains in several varieties of whole grain barley flour. Strains isolated from cereals were compared with one strain previously isolated from cereals and two commercial probiotic strains of *L. reuteri*, as well as one strain of *Lactobacillus plantarum* (control).

In Paper II, the experiments focused on improvement of some functional properties of *L. reuteri* DSM 17938, including tolerance to stress inflicted by repeated cycles of freeze-thaw and exposure to bile, as well as adhesion capacity to mucus. By implementing experimental evolution, subpopulations with improved functional properties were isolated and tested for the stability of these properties. Genome sequencing and proteomics were performed to identify mutations and changes in protein expression.

Further evaluations of the new variants were performed in Paper III. Experiments on other possible phenotypic changes, including growth kinetics, hydrophobicity, binding to epithelial cells, and also viability, stability and growth kinetics after lyophilisation (freeze-drying), were performed. Finally, the potential of the variants to interact with the host was compared with that of the wild-type strain in three cell culture models, including two epithelial cell models (IPEC-J2 and Caco-2), to study the effect on intestinal permeability, and one lymphocyte cell model (Jurkat), to study the antagonistic effect on the TRPV1 receptor involved in mediating visceral pain.

Results related to Paper I which need to be repeated, including evaluation of the protective effect of fermented barley on intestinal permeability and targeted chemical analysis to find possible production of certain bioactive tryptophan catabolites, are also reported in this thesis (section 3.2). The origin and name of strains and variants used in the thesis can be found in Table 1.

Table 1. *Genera, species, strain name, variant name and origin of bacteria used in this thesis*

Genus	Species	Strain name	Variant name	Origin
Lactobacillus	*reuteri*	DSM 17938	WT	Plasmid-cured variant of ATCC 55730 (Rosander *et al.*, 2008)
	reuteri		FT1	Freeze-thaw tolerance selection isolate
	reuteri	DSM 32847	FT2	Freeze-thaw tolerance selection isolate
	reuteri		BT5	Bile tolerance selection isolate
	reuteri	DSM 32846	BT6	Bile tolerance selection isolate
	reuteri	DSM 32848	MB4	Mucus binding selection isolate
	reuteri	DSM 32849	MFT3	Combination isolate, originally FT2 isolated during mucus binding selection
	reuteri	ATCC PTA 6475		Breast milk isolate (Spinler *et al.*, 2008)
	reuteri	LTH 5531		Sourdough isolate (Dal Bello *et al.*, 2007)
	reuteri	SU12-3		Buckwheat isolate (Pallin *et al.*, 2016)
	reuteri	SU18-3		Millet isolate (Pallin *et al.*, 2016)
Lactobacillus	*plantarum*	36E		Silage isolate (Johansson *et al.*, 1995)
Escherichia	*coli*	853/67		(Söderlind *et al.*, 1973)

2.1 Culture conditions and methods

Isolation of new strains from different cereals was achieved by incubating flour-water mixtures at 37 °C for 24 hours in a water bath without shaking (Paper I). The fermented mixtures were serially diluted in PBS (pH 7.4), plated on de Man, Rogosa and Sharpe (MRS) agar or Rogosa agar with added vancomycin (50 µg mL^{-1}), and incubated anaerobically at 37 °C and 45 °C, respectively. Single colonies were picked and transferred to new MRS plates with a numbered grid system for subsequent identification and characterisation.

Pre-cultures for growth and tolerance assays were inoculated in MRS broth and incubated at 37 °C overnight. For determining the bacterial densities and viable counts, bacteria in different liquid media were serially diluted and plated on MRS using the drop plate method (Hoben & Somasegaran, 1982), and incubated anaerobically at 37 °C overnight. Counted colonies from several 10-fold dilutions were multiplied by the dilution factor and averaged, in order to determine the number of colony-forming units (CFU) per mL of liquid culture. Growth studies were performed using six barley flours with slightly different carbohydrate composition (Table 2). Different flours were used to study the

importance of dietary fibre and starch content and composition on the growth and metabolism of *L. reuteri*. For this, 8 g of whole grain flour were mixed with 21 mL of sterilised deionised water. An overnight culture of each strain was added to the mixture and it was incubated in a 37 °C water bath for 24 hours, followed by backslopping (transfer of 1 % of the overnight culture to a fresh mixture). This procedure was repeated three times before bacteriological and chemical analysis.

Table 2. *Differences in nutritional content of the six barley varieties used in Paper I. The composition of dietary fibre (DF) and starch was analysed by the Food Science Unit, Department of Molecular Sciences, SLU, Uppsala*

		Fructan	Arabinoxylan	Total DF	β-glucan	Starch	Amylose
120	NGB 114602	0.9	7.3	18.9	4.4	57.4	27.1
155	SLU7	3.2	10.7	33	10.5	38.9	27.7
181	KVL 301	1	11.5	24.3	2.4	44.9	26
224	SW 28708	2.8	5.7	18.8	6.3	55.4	1.3
228	KARMOSÉ	2.2	8	22.7	5.1	49.7	40.7
249	GUSTAV	1.6	6.3	17.9	4.2	59.3	28.4

2.2 Identification and characterisation of strains

In Paper I, DNA from the fermented cereals was extracted according to the protocol for Gram-positive bacteria in the DNeasy Blood & Tissue Handbook (QIAGEN, Hilden, Germany) and analysed for the presence of *L. reuteri* DNA by polymerase chain reaction (PCR) using species-specific primers (L-reu 1 and L-reu 4) (Song *et al.*, 2000). Colonies from Rogosa plates with added vancomycin were identified by amplification and sequencing of 16S rRNA genes using the primers F8M (Edwards *et al.*, 1989) and 926r (Muyzer *et al.*, 1993). The PCR products were sequenced by Macrogen Inc. and isolates were identified at species level by a GenBank DNA search using the BLAST algorithm. Isolates identified as *L. reuteri* were grouped by genomic fingerprinting with rep-PCR, using GTG_5 primer (Versalovic *et al.*, 1994). Strains were further characterised using an assay for the production of reuterin (Rosander *et al.*, 2008). The genes encoding glycerol dehydratase, involved in reuterin production, and urease were detected by PCR using the primers pduCR/pduCF and ureCR/ureCF, respectively (Walter *et al.*, 2011). Multi-locus sequence analysis (MLSA) was also employed, using primers for fragments of seven housekeeping genes (*ddl*, *dltA*, *gyrB*, *leuS*, *pkt*, *rpoA* and *recA*) (Oh *et al.*, 2009). With this procedure, strains can be further characterised by concatenating and aligning the genes using Geneious R6, and comparing strains to previously

isolated strains of different animal origin. A phylogenetic tree can then be made to illustrate the similarities between the strains.

To determine whether the new variants of DSM 17938 in Paper II were in fact DSM 17938 and not contaminants, a PCR using strain-specific primers (LR1/1694f and LR1/1694r) (Vestman *et al.*, 2013) was performed.

2.3 Chemical analyses

For the metabolic profiling used to group barley-bacteria combinations in Paper I, proton nuclear magnetic resonance (1H-NMR) was used. As part of the thesis work, a targeted chemical analysis was also performed using quadrupole time of flight-liquid chromatography mass spectrometry (QTOF-LCMS). The aim was to find tryptophan catabolites produced in the samples studied in Paper I and in additional fermentations.

2.3.1 Proton nuclear magnetic resonance (1H-NMR)

Triplicate samples from fermented barley slurries with each combination of barley-bacteria were centrifuged and sterile-filtered and the pH was adjusted to 7.4 by dilution in phosphate buffer and addition of NaOH. Buffered samples were stored at -20 °C and thawed before analysis with NMR. After addition of D_2O and an internal reference, the spectra were recorded on a Bruker Avance-III 600 MHz spectrometer. Peaks in the NMR spectra were identified and concentrations were determined using Chenomx NMR suite 7.5 (Chenomx Inc., Edmonton, AB, Canada), the Human Metabolome Database (www.hmdb.ca) and the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu/metabolomics/).

2.3.2 Quadrupole time of flight-liquid chromatography mass spectrometry (QTOF-LCMS)

Slurries from different barley varieties fermented by *L. reuteri* DSM 17938 and ATCC PTA 6475 were centrifuged and the supernatants collected. The supernatant was filtered through a 5-µm Acrodisc syringe filter (Pall Life Sciences, Portsmouth, UK) followed by a 0.45-µm sterile filter (Filtropur S, Sartstedt, Nümbrecht, Germany) and the samples were stored at -20 °C. Thawed filtered samples were mixed with methanol at a ratio of 4:6 (sample:methanol) to reach a final volume of 500 µL. The mixtures were vortexed for 1 minute before being centrifuged at 10 000 x *g* for 10 min. Then 100 µL of the mixture were transferred to a LC-vial and analysed by LC/Q-TOF/MS. Standards for

serotonin, melatonin, tryptophan, tryptamine, indole-3-acetate, indole-3-acetic acid and kynurenine were also prepared in two concentrations (100 and 1000 nM).

The chromatography part of the analysis was performed on an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA), where the aquatic phase A consisted of 10 mmol L^{-1} ammonium formate with 0.1% formic acid (v/v). Mobile phase B was made from acetonitrile with 0.1% formic acid (v/v). From these phases a gradient was made, the percentage indicating mobile phase B: 0 min 95%, 0.5 min 95%, 10.5 min 40%, 15 min 40%, 17 min 95% and 32 min 95%. Extracts were injected onto a Waters Atlantis HILIC Silica column (3 µm, 2.1 x 150 mm), containing a 10-mm guard column (same composition). The column oven was kept at 30 °C and the flow rate over the column was 0.25 mL min^{-1}. A time-of-flight mass spectrometer (Bruker maXis impact, Bruker Daltonics, Bremen, Germany) was operated in positive ionisation mode with a plate offset voltage of 500 V and a capillary voltage of 4kV. For the removal of solvents from the samples, nitrogen gas heated to 200 °C was administered at a rate of 8 L min^{-1} with a nebuliser pressure of 2 bar. The digitiser sample rate was set at 4 GHz and profile sample spectra were collected at a rate of 1 Hz. Data were processed using Compass DataAnalysis 4.1 (Bruker Daltonics, Bremen, Germany). Chromatograms were smoothed by one cycle of Gaussian smoothing with a width of 2 seconds prior to area calculation. Relative quantification of selected metabolites was performed by using the area of extracted ion chromatograms based on compound accurate masses (± 0.01 m/z).

2.4 Strain improvement

The experiments on strain improvement (Papers II and III) were performed using different assays, including freeze-thaw tolerance, bile tolerance and mucus binding capacity.

2.4.1 Freeze-thaw tolerance

Lactobacillus reuteri strain DSM 17938 was evaluated for its tolerance to repeated cycles of freezing at -50 °C followed by thawing at 37 °C (Paper II). After each cycle, samples were taken for viability counts where 10-fold dilution series were plated (drop plate method) on MRS agar. Figure 2 illustrates the workflow of the assay. After incubation of plates, colonies were picked from different time points, re-streaked on MRS plates and frozen as new variants. These variants were later tested in the same assay and compared against wild-type DSM 17938. This method is based on an unpublished assay procedure developed by Armando Hernández Garcia, SLU, Uppsala.

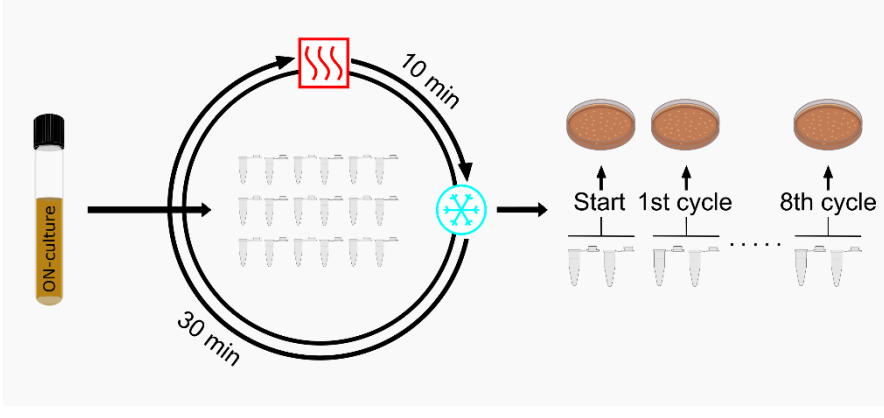

Figure 2. Overview of the freeze-thaw tolerance assay. Overnight cultures of *Lactobacillus reuteri* strain DSM 17938 wild type and new variants with improved functional properties were exposed to repeated cycles of freezing (-50 °C) and thawing (37 °C). Viable counts were determined by the drop plate method after each cycle.

2.4.2 Bile tolerance

Strain DSM 17938 was also evaluated for its tolerance to bile (Paper II). The growth medium of an overnight liquid culture was replaced with fresh MRS broth containing 0.5% (w/v) porcine bile and incubated at 37 °C for four hours. Viable count was determined immediately and then every 30 minutes during exposure to bile medium, by plating (drop plate method) 10-fold dilutions of the culture (Figure 3). After incubation of the plates, colonies were picked, re-streaked and later frozen as new variants. Variants were tested using the same assay and compared with wild-type DSM 17938.

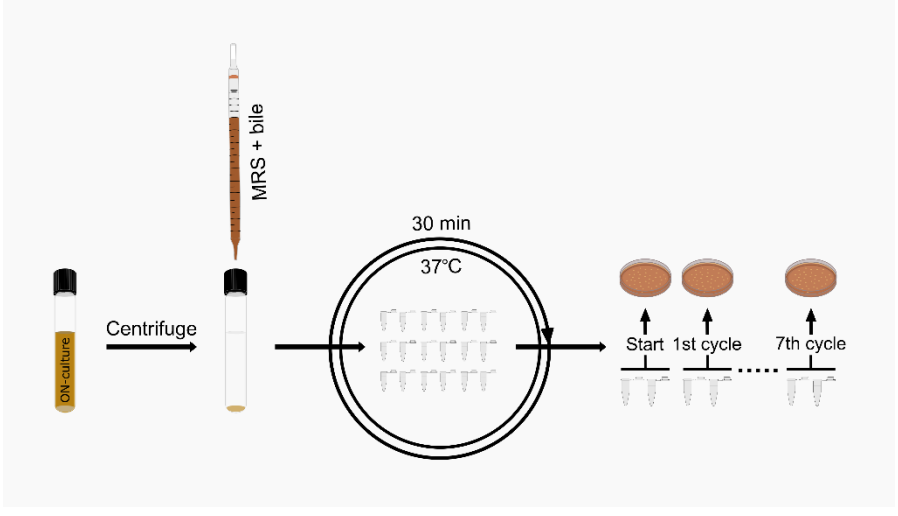

Figure 3. Overview of the bile tolerance assay. Overnight cultures of *Lactobacillus reuteri* strain DSM 17938 wild type and new variants with improved functional properties were exposed to 0.5% (w/v) porcine bile for 240 minutes. Viable counts were determined by the drop plate method and analysed every 30 minutes.

2.4.3 Mucus binding capacity

The mucus binding capacity of DSM 17938 was evaluated (Paper II) using a modified version of an existing method (Roos & Jonsson, 2002) (see Figure 4). In brief, microtitre wells were coated with porcine mucus and then washed and blocked for unspecific binding. Overnight cultures of DSM 17938, labelled with carboxyfluorescein diacetate (cFDA), were added to the wells and incubated at 37 °C for four hours. After washing of the wells, the number of attached bacteria was determined by inverted microscopy, followed by addition of lysis buffer (1% sodium dodecyl sulfate in 0.1M NaOH) and analysis of fluorescence intensity using a plate reader. Isolation of variants with improved mucus binding capacity was achieved by skipping the lysis step and instead adding fresh MRS

broth, incubating the wells for 24 hours and plating on MRS agar. Colonies from this enrichment procedure were re-streaked and frozen as new variants of wild-type DSM 17938.

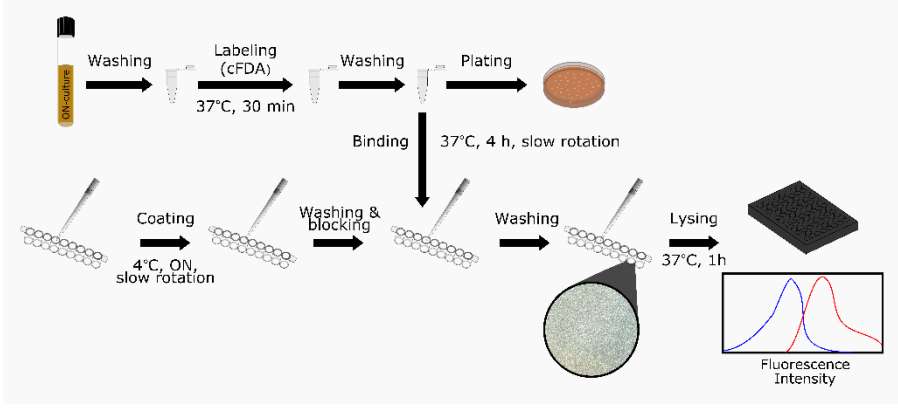

Figure 4. Overview of the mucus binding assay. Microtitre plates were coated with porcine mucus, washed and blocked, followed by binding with cFDA-labelled overnight cultures of *Lactobacillus reuteri* strain DSM 17938 wild type and new variants with improved functional properties. Unbound bacteria were washed away and binding capacity was determined by inverted microscopy and by analysing fluorescence intensity.

2.4.4 Stability test

The stability of the improved attributes was tested by performing the same assays after 1, 3, 7 and 15 re-inoculations of the variants in MRS broth. The stability of a selected few of the new variants from the freeze-thaw tolerance, bile tolerance and mucus binding assays was tested.

2.4.5 Hydrophobicity

Changes in hydrophobicity of new variants of DSM 17938 were analysed by a procedure based on an existing method (Kos *et al.*, 2003). In brief, overnight cultures were centrifuged, washed and re-suspended in PBS, and then the optical density (OD) measured at 600 nm (OD_{600}) was adjusted to 0.5. To 3 mL of culture, 1 mL of xylene was added and the cultures were incubated at room temperature. This was followed by vortexing and additional incubation at room temperature. The optical density was measured before addition of xylene and also in the aqueous phase after xylene addition, in order to determine the partitioning of the bacteria and thereby the degree of hydrophobicity. Xylene was chosen because of its apolar attributes, which is considered to make it a good solvent for evaluating cell surface hydrophobicity (Kos *et al.*, 2003).

2.4.6 Analysis of growth kinetics by optical density

To determine growth kinetics of strains, changes in optical density were measured over a time period of 24 hours, using the BioScreen C microbiological growth monitoring system (Oy Growth Curves Ab Ltd, Helsinki, Finland). This machine automatically measures optical density at different wavelengths, can shake the plates and has temperature control. By adding bacteria to each well and adjusting the starting optical density, the lag phase, exponential phase and stationary phase can be determined during different conditions. In Paper III, this method was used to determine differences between wild-type DSM 17938 and new variants. Growth kinetics were studied for overnight cultures grown in MRS broth and for lyophilised cultures.

2.4.7 Lyophilisation experiments

Lyophilisation of wild-type DSM 17938 and the new variants was performed to investigate the correlation between increased freeze-thawing tolerance and lyophilisation survival ability and stability (Paper III). It was also used to investigate whether the survival ability of the other variants were similar to DSM 17938 or whether any changes had occurred.

Pre-cultures for each variant and DSM 17938 were performed in 9 mL MRS broth incubated at 37 °C overnight. On the following day, the entire volume was added to 400 mL of fresh MRS broth and incubated at 37 °C overnight. The 400 mL cultures were concentrated 20-fold in a 10% sucrose solution to a final volume of 20 mL. Aliquots of 1 mL were transferred to vials and these were placed in the lyophilising apparatus (Epsilon 2-6D LSCplus, CHRIST, Osterode, Germany) and run with for 44 hours under vacuum with a starting temperature of -50 °C. Viable counts were determined before and after lyophilisation, by the drop plate method. An accelerated stability experiment was performed by incubating the lyophilised products at 37 °C and checking viable counts every week for 5 weeks.

2.4.8 Genomics

Genome sequencing was performed by Jennifer Spinler at Baylor College of Medicine, Houston, Texas, USA. To determine whether any changes in the genome had occurred during improvement of DSM 17938, whole-genome shotgun sequencing was performed on an Illumina MiSeq instrument (Paper II). Changes in sequences, including exchanges in base pairs, inserts or deletions, in the variants were mapped using the reference genome for DSM 17938.

2.4.9 Proteomics

Changes in protein expression of wild-type DSM 17938 compared with the new variants were analysed by Nanoxis Consulting AB (Gothenburg, Sweden). In brief, cell pellets containing approximately 9.7 $\log_{10}$ bacterial cells were suspended in PBS and gently inverted during a 20-min incubation at room temperature. The suspensions were further diluted (10-fold) before being transferred to a vial containing glass beads. Bacterial cells were mechanically lysed using a TissureLyser (Qiagen) and then frozen until analysis. Sodium deoxycholate (SDC) and trypsin were added to the samples, which were incubated for eight hours at 37 °C. Neat formic acid was then added, followed by centrifugation and collection of supernatant. The samples were labelled using tandem mass tag (TMT), and analysed by high-resolution Orbitrap LC-MS/MS. The protein sequences predicted from the genome sequence of DSM 17938 were used to identify the peptides.

2.5 Evaluation of host interactions *in vitro*

Evaluation of the potential of new probiotic candidate strains often begins with simple *in vitro* models, evaluating tolerance to *e.g*. low pH, bile, mucus binding. This is usually followed by evaluation of host interactions using different cell culture models. In this thesis, cell culture models were used to study the effect of *L. reuteri* on intestinal permeability and the interactions with the host's own cells of different types, both epithelial and immune cells (Paper III). One small intestinal cell line (IPEC-J2) and one large intestinal cell line (Caco-2) were used, in order to study possible similarities in effect on different parts of the intestine.

2.5.1 Intestinal permeability models

IPEC-J2

IPEC-J2 is a non-transformed epithelial cell line derived from the mid-jejunum of a neonatal piglet (Berschneider, 1989), and is a well-studied model as regards cell interactions with pathogenic bacteria and viruses. In Paper III, these cells were used to study the protective effect of wild-type DSM 17938 and new variants on epithelial permeability. They were also used to study the protective effect of the supernatants collected during fermentation of barley in Paper I (the results presented are preliminary and not published elsewhere).

IPEC-J2 cells were cultivated in flasks with Dulbecco's modified Eagle's medium/F12 Ham (DMEM/F-12) containing 0.12% sodium bicarbonate, 15 mM HEPES, pyridoxine and L-glutamine, with 5% foetal bovine serum (FBS), 0.5mM sodium pyruvate and antibiotics (penicillin and streptomycin). Cells were kept in an incubator at 37 °C in a humidified atmosphere of 5% CO_2. Cells were maintained by changing the medium every 2-3 days and passaging every 4-7 days. Experiments were performed on cells seeded on collagen-coated membrane filters, kept for 11-12 days with change of medium every other day, the last medium change, to antibiotic-free medium, being made 24 hours before the experiment. Filters were pre-treated with *L. reuteri* for six hours, followed by six hours of challenge with pathogenic enterotoxigenic *Escherichia coli* (ETEC), known for having a disruptive effect on epithelial integrity. Permeability of the seeded monolayer of IPEC-J2 cells was measured in two ways, by transepithelial electric resistance (TEER) and by measuring the leakage of a high molecular weight fluorescent marker (fluorescein isothiocyanate (FITC)-dextran 4kDa) from the apical to the basolateral side.

Caco-2

Caco-2 is a human intestinal cell line derived from a human colon adenocarcinoma (Fogh *et al.*, 1977). It is mainly used since the cells can develop morphological characteristics of normal enterocytes and it is considered a good model for intestinal epithelial permeability.

In Paper III, the Caco-2 cells were used as a complementary cell line for studying cell adherence. The protocol for managing the cells was similar to that used for the IPEC-J2 cells with one exception that the amount of FBS was kept at 10% during cultivation of the cells and during all experiments.

2.5.2 TRPV1 receptor activation in Jurkat cells

The transient receptor potential vanilloid subtype 1 (TRPV1) receptor is a non-selective cation channel in peripheral sensory neurons. It is involved in pain sensing and, when activated, leads to an increased influx of calcium ions (Ca^{2+}). This channel can also be activated by *e.g*. increased temperature, acidic or basic pH and by endovanilloids such as capsaicin (Pecze *et al.*, 2013). Jurkat cells are a transformed human T-cell line obtained from leukaemic cells (Martin *et al.*, 1981). Studies have confirmed that the plasma membrane of Jurkat cells contains TRPV1 (Bertin *et al.*, 2014), which makes this cell line a good model for studies on activation of TRPV1.

Jurkat cells were cultivated in flasks with Roswell Park Memorial Institute (RPMI) medium containing 10% FBS, L-glutamine, β-mercaptoethanol and

antibiotics (penicillin and streptomycin). The cells were kept in an incubator at 37 °C in a humidified atmosphere of 5% CO_2 and passaged every three days, when full confluence was achieved. On the day of the experiment, the medium was changed to an antibiotic-free medium, before addition of fluorescent dyes. Two dyes were added to study the change in ratio between intra- and extracellular Ca^{2+} levels, *i.e.* the influx of Ca^{2+}. After one hour of incubation under normal incubation conditions, cells were spun down and new medium was added along with *L. reuteri* at different bacteria:Jurkat cell ratios. Following another one-hour incubation, the cells were spun down and the medium was replaced with PBS containing Ca^{2+}. Capsaicin was added to each tube, followed by immediate analysis of ratiometric calcium flux by flow cytometry (BD FACSCelesta, BD Bioscience).

2.6 Statistical analysis

Different statistical analyses and evaluations were performed depending on the type of experiment. In Paper I, one-way and two-way analysis of variance (ANOVA) with Student's t-test was performed using JMP Pro 11 (SAS, Cary, NC) to determine differences in the bacterial densities of the strains cultured in slurries made from all barley varieties with the two different pre-treatments. Principal component analysis (PCA) was used to determine possible correlations between specific sugar contents and bacterial densities and a decline in pH (JMP Pro 11), and also to find possible groupings from the metabolic profiling of the different barley-bacteria combinations using Simca 13 (Umetrics AB, Umeå, Sweden) in Paper I. A complex statistical model, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA), was also used to try to group these metabolic profiles, with Pareto scaling used for the OPLS-DA modelling. Results from the experiments described in Papers II and III were analysed by one-way ANOVA followed by *post hoc* pair-wise comparison using Tukey's t-test.

3 Results and discussion

3.1 Growth and metabolism of *L. reuteri* in whole grain barley flour (Paper I)

Paper I examined whether cultivation of *L. reuteri* in cereal substrates could induce or increase the production of bioactive compounds. Isolation of new strains of cereal origin was performed to determine whether ecological adaptations existed in such strains, evaluated as differences in growth and metabolism compared with strains of other origin. *Lactobacillus reuteri* has previously been shown to be a stable and sometimes dominant member of high temperature type II sourdoughs (Corsetti & Settanni, 2007; Gänzle & Vogel, 2003), even though it is mainly associated with the gastrointestinal tract, vagina, breast milk and oral cavity of warm-blooded animals. A possible animal origin of the strains isolated from sourdough was first suggested in 2012 (Su *et al.*, 2012), almost two decades after some of the earliest isolations (Vogel *et al.*, 1994). In Paper I, new strains of *L. reuteri* were isolated from three cereals, namely barley, buckwheat and millet. It has been shown by Su *et al.* (2012), using MLSA and construction of a phylogenetic tree, that sourdough strains are positioned in clades with strains of either rodent or human origin. The strains isolated in Paper I were reuterin-negative and urease-positive, characteristics usually seen in strains of rodent origin (Frese *et al.*, 2011; Walter *et al.*, 2011). When a MLSA was performed on the newly isolated strains in Paper I, as in previous studies it revealed that strains of cereal origin cluster together with rodent strains, in this case in clade I and III of the phylogenetic tree (Figure 5). While the sourdough strains may be of rodent origin, other studies have shown that these strains have adapted to this new ecological environment, with an equal or even higher competiveness in sourdoughs than strains of rodent origin (Zheng *et al.*, 2015). Certain activities also seem to be unique among these sourdough-adapted strains, such as the presence of a operon of genes connected to the

production of reutericyclin, an antibiotic active against a number of different Gram-positive bacteria (Gänzle & Vogel, 2003; Vogel *et al.*, 1999). The production of reutericyclin has been suggested to contribute to the competiveness of those strains in sourdoughs (Gänzle & Vogel, 2003).

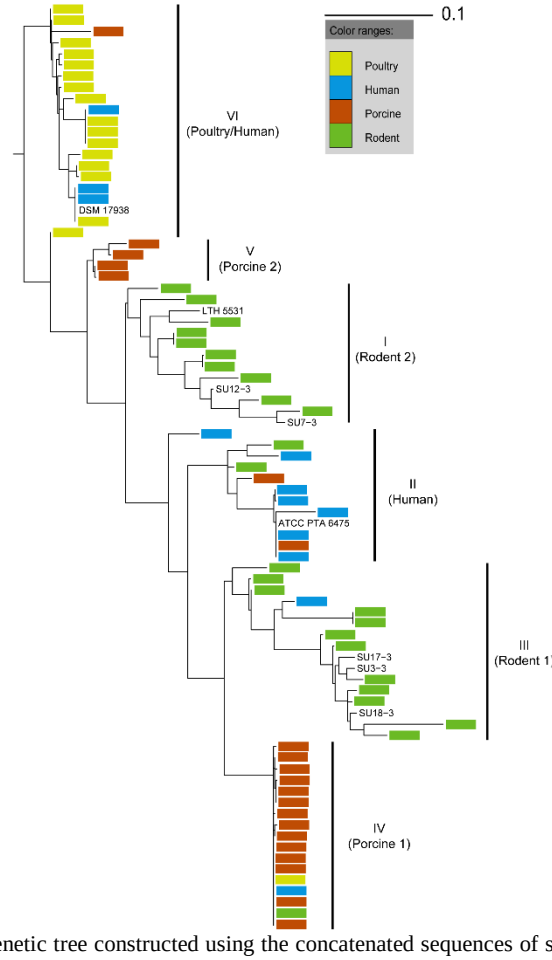

Figure 5. Phylogenetic tree constructed using the concatenated sequences of seven housekeeping genes from 84 strains of *Lactobacillus reuteri* by multi-locus sequence analysis (MLSA). Strains isolated (SU3-3, SU7-3, SU12-3, SU17-3 and SU18-3) and used (LTH 5531, DSM 17938 and ATCC PTA 6475) in Paper I are marked with strain number instead of colour coding.

To study possible adaptations of the strains isolated in this thesis work, the growth and metabolism of two of the isolated sourdough strains from Paper I (SU12-3 and SU18-3) were studied in six different barley flours and compared with those of another sourdough strain (LTH 5531) and two strains of human origin (DSM 17938 and ATCC PTA 6475). To further push the strains to reveal differences, they were cultivated in both untreated and heat-treated whole grain

barley flour. The heat treatment of flour was performed to inactivate endogenous enzymes and microorganisms which could aid the growth of the strains. The results revealed higher bacterial counts in untreated compared with heat-treated flours, showing the importance of such endogenous enzymes for the growth of *L. reuteri* in cereal substrates. Only minor differences in bacterial counts were seen between the strains in untreated barley flour. In heat-treated flour, DSM 17938 generally had lower counts than the other *L. reuteri* strains, while ATCC PTA 6475 generally had higher counts. In untreated flour, the bacterial counts in the slurries with the six barley varieties were similar for all strains, only varying by 0.5 $\log_{10}$ CFU g^{-1}. In heat-treated flour the differences were greater, at least for DSM 17938 with bacterial counts ranging from 7.3-8.8 $\log_{10}$ CFU g^{-1}, while the other strains had similar differences (0.4-0.7 $\log_{10}$ CFU g^{-1}) as in untreated flour. While the differences was not great for most bacterial strains, they generally grew better in barley variety 224, while they had slightly lower bacterial densities in slurries of varieties 120 and 249. Metabolic profiling by 1H-NMR was performed to visualise potential differences in metabolism seen for the different combinations of strains and barley varieties. Multivariate statistical analysis (OPLS-DA) of the metabolic profiles of strains from untreated flour revealed that the profiles of the sourdough strains formed clusters separated from the two strains of human origin (Figure 6).

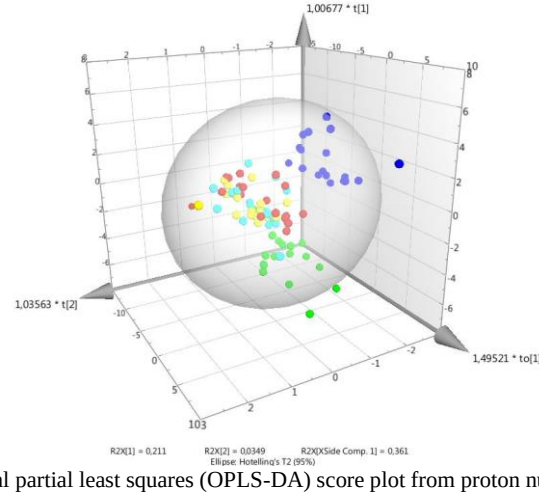

Figure 6. Orthogonal partial least squares (OPLS-DA) score plot from proton nuclear magnetic resonance (1H-NMR) spectrum profiles of all different fermented untreated barley flours. Class ID (strain of *Lactobacillus reuteri*) marked as follows: ATCC PTA 6475 = green; DSM 17938 = blue; LTH 5531 = red; SU12-3 = yellow; SU18-3 = cyan.

The metabolites mainly responsible for the separation into clusters in untreated flour were succinate, lactate, acetate and ethanol. Barley fermented by strain DSM 17938 had lower concentrations of succinate than barley fermented by the other reuteri strains, while barley fermented by ATCC PTA 6475 had lower concentrations of acetate, lactate and ethanol than found with most other strains. Lower concentrations of succinate were also seen in heat-treated flours fermented by DSM 17938, while only minor differences in other major metabolites were seen. The lower succinate concentration could indicate that DSM 17938 uses other electron acceptors than fumarate and malate, or that the metabolic reaction continues to form lactate from malate, using malolactic enzymes (Stolz *et al.*, 1995).

Although the bacterial counts of the sourdough strains were not higher in barley flour than those of the strains of human origin, the differences observed in metabolic profiles might indicate a certain degree of adaptation to this ecological niche, as suggested in previous studies (Zheng *et al.*, 2015). Tests were made for correlations between bacterial densities and nutritional content of the barley varieties, based on information on the nutritional content of the different barley flours previously obtained by colleagues at the Department of Molecular Sciences, as well as data on the sugar and amino acid content provided by 1H-NMR. No such correlations were found, but the amount of information about each barley variety was very limited and differences in content that might explain differences in growth and metabolism could have been missed. Only one possible correlation was found, and that was with tryptophan content. In heat-treated barley flour, the varieties (var.) with the highest bacterial densities (var. 181 and 224) had the highest tryptophan concentrations, while the varieties with the lowest bacterial densities (var. 120 and 249) had lowest tryptophan concentrations. The levels of tryptophan were also decreased in barley varieties 181 (Figure 7) and 224 fermented by *L. reuteri* and *L. plantarum*, but not in variety 120. These correlations were seen for all but one bacterial strain (ATCC PTA 6475).

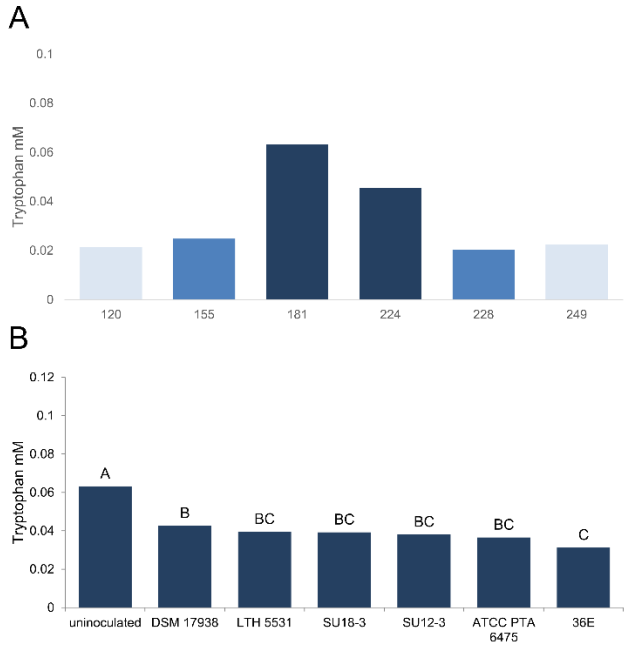

*Figure 7. (*A) Tryptophan levels (mM) in the different heat-treated barley flours and (B) changes in tryptophan levels after fermentation by *Lactobacillus reuteri* and *L. plantarum* (36E) in barley variety 181. Mean values for three biological replicates. Bars with different letters are significantly different (p<0.05).

3.2 Production of AhR ligands and effect of fermented barley slurries *in vitro* (unpublished)

Lactobacillus reuteri has been shown to utilise tryptophan under certain conditions, leading to the production of bioactive derivatives, some of which are called aryl-hydrocarbon receptor (AhR) ligands (Zelante *et al.*, 2013). Those authors postulated that utilisation of tryptophan and production of its derivatives mainly occur under conditions with high tryptophan levels and low carbohydrate levels (carbohydrate starvation). Since the levels of tryptophan were found to decrease in some of the fermented barley varieties in Paper I and since the availability of fermentable sugars was low in the heat-treated barley flours, more targeted chemical analyses were performed. QTOF-LCMS was utilised, since it can detect lower concentrations of compounds than 1H-proton NMR. Identification of specific compounds can also be made more accurate by adding standards of known concentrations for each targeted compound. The results revealed that the levels of two AhR ligands (tryptamine and indole-3-aldehyde) had increased in heat-treated flour (barley variety 224) fermented by *L. reuteri* DSM 17938 and 6475 compared with unfermented flours (Figure 8).

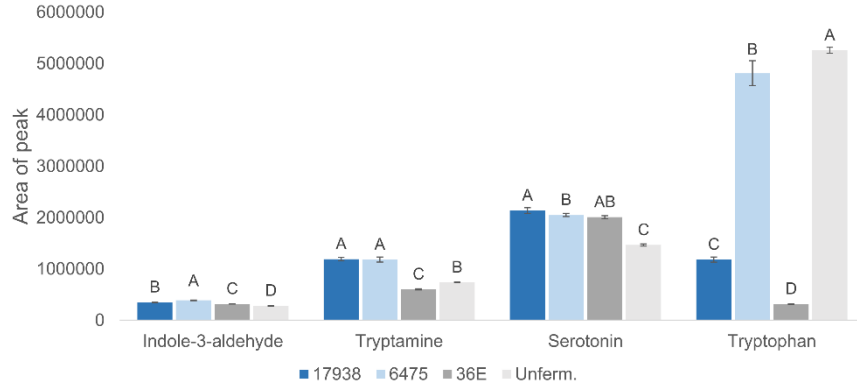

Figure 8. Results (mean ±SEM) of chemical analysis by quadrupole time of flight-liquid chromatography mass spectrometry (QTOF-LCMS). Area of identified peaks for tryptamine, serotonin, indole-3-aldehyde and tryptophan in whole grain flour from barley variety 224 fermented by *Lactobacillus reuteri* strains DSM 17938 and ATCC PTA 6475, compared with fermentation by *L. plantarum* 36E and unfermented barley flour. Bars with different letters are significantly different (p<0.05).

Serotonin, another tryptophan derivative, was also increased in the same samples and in flours fermented by *L. plantarum* 36E. Increases in tryptamine were not seen for *L. plantarum*. For DSM 17938, the increases seen in serotonin and tryptamine were correlated with a decrease in tryptophan, but only for certain barley flours. Such correlations were not seen at all for ATCC PTA 6475, where no or only small decreases in tryptophan were seen, although increases in tryptophan derivatives were detected. Similar results, showing increases in tryptamine and serotonin, were seen in three consecutive experiments, indicating an increase in these compounds in heat-treated barley flour during fermentation with *L. reuteri*. In an attempt to demonstrate actual production, and not merely solubilisation, of those compounds in the supernatants, a labelled tryptophan (L-tryptophan-15N2) was added to the flour-water mixtures during the fermentation with *L. reuteri*. However, none of the labelled derivatives of tryptophan was detected after fermentation, even though the levels of labelled tryptophan decreased during fermentation of barley varieties 224 and 120 with strains DSM 17938 and ATCC PTA 6475. While the levels of both tryptamine and serotonin increased in barley variety 224, analysis of all six varieties revealed that tryptamine levels were increased in all varieties (Figure 9). Serotonin was also increased during the fermentation of most barley varieties by both DSM 17938 and 6475, compared with the unfermented flour-water mixtures. The changes in tryptamine levels were highest for barley variety 224. The same was true for

serotonin levels fermented by DSM 17938, but not for ATCC PTA 6475, where the levels were slightly higher in barley variety 155. Interestingly, the amount of soluble tryptophan did not change much during the fermentation with ATCC PTA 6475, the levels being close to those in the unfermented flour-water mixture for all barley varieties. For DSM 17938, the levels of tryptophan were significantly decreased for two of the barley varieties (var. 155 and 224).

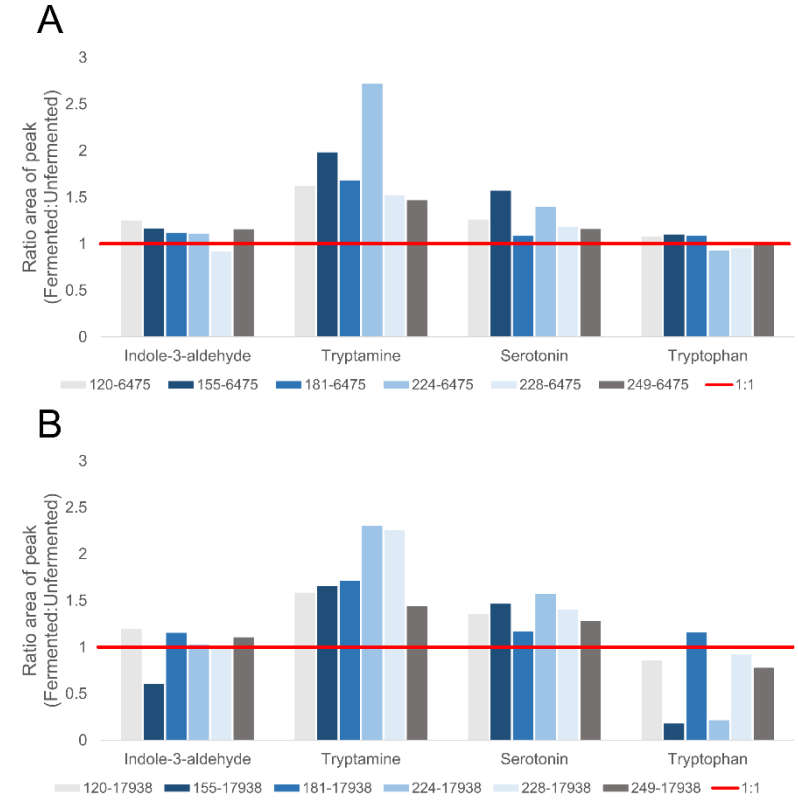

Figure 9. Results of chemical analysis by quadrupole time of flight-liquid chromatography mass spectrometry (QTOF-LCMS). Fermentation of six different varieties of barley (120, 155, 181, 224, 228 and 249) by *Lactobacillus reuteri* strains DSM 17938 and ATCC PTA 6475. Bars show the ratio between fermented and unfermented samples.

The importance of AhR ligands in immune responses, as mentioned in previous sections, led to the hypothesis that *L. reuteri* can utilise tryptophan to produce AhR ligands during culture in barley flour slurry, which in turn could have a protective effect on intestinal epithelial permeability. Evaluations *in vitro* of some of the fermented barley slurries were performed using the intestinal epithelial permeability model with IPEC-J2 cells. The results showed that

supernatants from one of the combinations of bacteria and heat-treated barley flour (120-6475) could have a protective effect on intestinal epithelial cell layer integrity (Figure 10). No dose-dependent effects were seen in a follow-up experiment, when the amount of added supernatant was increased from 20 µL to 40, 80 and 150 µL. The protective effect was only seen for leakage of the fluorescent macromolecule (FITC-dextran, 4 kDa) and not for transepithelial resistance (TEER). No significant delay in decrease in TEER was seen for any of the pre-treatments. However, the effect of barley:*L. reuteri* combinations did not correlate to content of AhR ligands, since the best combination (120-6475) had one of the lowest levels of tryptamine. The protective effect of the supernatant was not as significant as that of the live bacteria (DSM 17938 and ATCC PTA 6475), indicating either that the concentrations of the bioactive compounds in the supernatants were too low, or that the mechanism behind the protective effect in this model is not linked to those bioactive compounds.

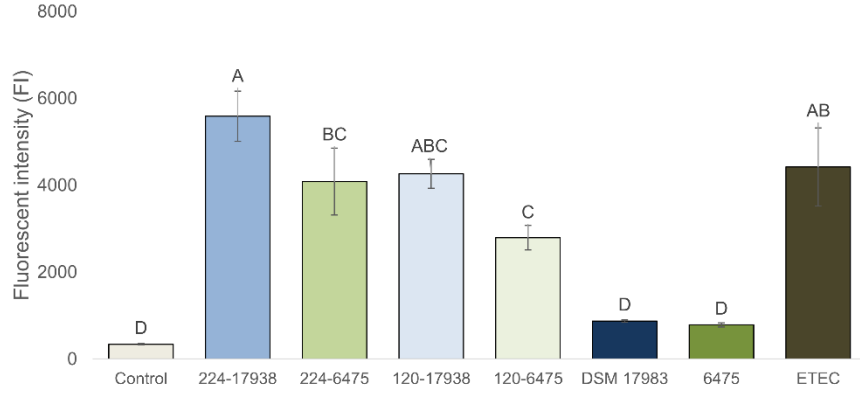

Figure 10. Results (mean ± SEM) of tests on intestinal epithelial integrity model (IPEC-J2), showing leakage of fluorescent molecules (FITC-dextran) across the monolayer. Bars with different letters are significantly different (p<0.05).

3.3 Improvement of DSM 17938 by experimental evolution (Papers II and III)

In Paper II, the focus was on improving certain functional properties of *L. reuteri* DSM 17938 using the experimental evolution approach. Properties to improve were chosen based on previously observed limitations of this strain compared with others. One crucial property for a commercially produced probiotic strain is the survival ability during lyophilisation (freeze-drying), since most products are administered in this form. A high percentage of viable bacteria after

lyophilisation would mean higher counts in each dose and possibly a greater chance of exerting an effect in the gastrointestinal tract. Based on observations made in our laboratory by Garcia *et al.* (unpublished), there is a correlation between tolerance to repeated cycles of freezing-thawing and survival ability during lyophilisation for DSM 17938. Similar correlations have been observed for *Lactobacillus plantarum*, where a strain with higher freeze-thaw tolerance also had higher survival ability during lyophilisation (Schoug, 2009). Studies on *Bifidobacterium longum* pre-treatment with salt show that it can enhance the tolerance to freeze-thaw and lethal heat stress (Crittenden, 2004). This indicates that the induction of stress proteins during the fermentation of a probiotic strain could improve survival ability during other types of stress, during *e.g.* manufacture, storage and gastrointestinal transit. To test this hypothesis, new variants with higher freeze-thaw tolerance were isolated during assays and compared with the wild type. Two isolates (FT1 and FT2) were used for further analyses, of which FT1 formed normal-sized colonies on MRS plates while FT2 formed slightly smaller colonies. Comparing the freeze-thaw tolerance of these two variants to wild-type DSM 17938 revealed that both had an improvement in tolerance, with FT2 being the slightly better variant. The new variants of DSM 17938 had a lower decrease in viable counts by the end of the assay, approximately 1.7 $\log_{10}$ CFU mL^{-1} compared with 2.6 $\log_{10}$ CFU mL^{-1} for the wild-type DSM 17938 (Paper II).

Two other properties believed to be important for a probiotic bacterium to exert a positive effect on the host are bile tolerance and mucus binding capacity. In order for probiotic bacteria to metabolise and be active in the intestine of the host, they need to survive the passage from the oral cavity down to the small and large intestine. Although DSM 17938 has been shown to be one of the most potent probiotic bacteria in clinical studies, the strain has a relatively low bile tolerance *in vitro* (Whitehead *et al.*, 2008). The poor tolerance to porcine bile seen in Paper II has not been reported previously. Previous studies investigating the tolerance to bovine bile have only revealed poor tolerance of DSM 17938 during the exponential phase, while it was found to tolerate and even grow during exposure to bile concentrations up to 5% after reaching the stationary phase (Rosander *et al.*, 2008; Whitehead *et al.*, 2008). However, bovine bile has been shown to be less inhibitory than porcine bile on other species of *Lactobacillus* (Dunne *et al.*, 2001; Grill *et al.*, 2000). Similar results have been reported for *Helicobacter hepaticus*, *i.e.* less inhibition during incubation with bovine bile salts compared with porcine and human bile salts (Okoli *et al.*, 2012; Okoli *et al.*, 2010). The lower inhibition of bovine bile compared with porcine bile on other bacteria, and especially other lactobacilli, suggests that porcine bile might be a better choice when evaluating human bile tolerance, since some

studies claim that porcine and human bile are more similar in inhibitory effect and in composition (Begley *et al.*, 2005). The low tolerance to 0.5% (w/v) porcine bile of DSM 17938 in Paper II was significantly improved through experimental evolution (Figure 11).

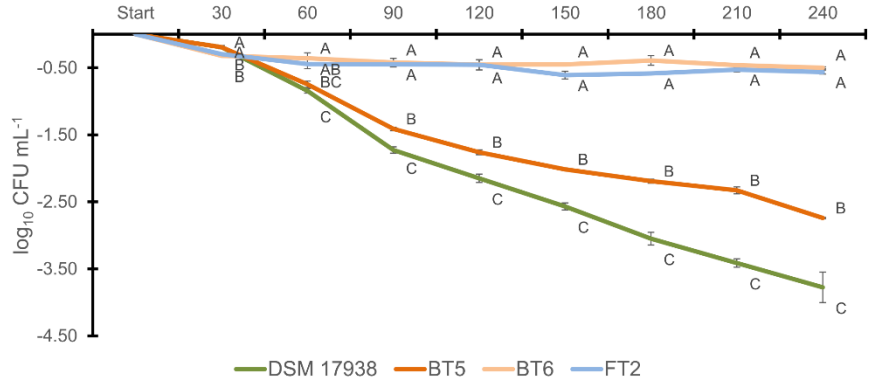

Figure 11. Decrease in viable counts, expressed as $\log_{10}$ CFU mL^{-1} (mean ± SEM), of *Lactobacillus reuteri* strain DSM 17938 wild type and variants BT5, BT6 and FT2 during exposure to 0.5% (w/v) porcine bile for 240 minutes. Marker points with different letters are significantly different (p<0.05).

Several new variants of DSM 17938 were isolated during the bile tolerance assay, and two were chosen for additional analyses. These were BT5, which formed normal-sized colonies, and BT6, which formed small-sized colonies. These two variants and variant FT2 were tested again and compared with wild-type DSM 17938. The results revealed that two of the variants (BT6 and FT2) had a decrease in viable counts of approximately 0.5 $\log_{10}$ CFU mL^{-1}, compared with 3.5 $\log_{10}$ CFU mL^{-1} for the wild type.

The genome analysis performed on variants FT2 and BT6 only revealed changes for FT2 compared with wild-type DSM 17938. An insertion of one nucleotide that caused a frameshift in a gene encoding a glycosyltranferase (*wcaA*) was detected. This gene is truncated in wild-type DSM 17938 and the insertion of the nucleotide seems to have made the gene intact again. Little is known about the function of WcaA in lactobacilli, but it is suggested to play a role in fatty acid or lipid synthesis (Yang *et al.*, 2017). The proteomic analyses of the two variants revealed more changes compared with wild-type DSM 17938. The results for the freeze-thaw tolerant variant FT2 and the bile tolerant BT6 were similar, and changes were mainly seen as protein downregulations in an operon of genes (*acc*, *fabD*, *acp*, *fabH*, *fabG*, *fabF*, *fabZ* and *fabI*) all related to type II fatty acid synthesis (Zhang & Rock, 2008). Changes in the gene sequence and expression of proteins related to fatty acid synthesis could indicate

possible changes in the lipid composition of the cell membrane. Changes in fatty acid composition, causing changes in cell membrane fluidity, have been observed in Gram-positive bacteria during different kinds of stress (Pepi *et al.*, 2008; Williams *et al.*, 2007; Nielsen *et al.*, 2005). In Paper II, induction of a protein belonging to the family of MFS transporters was also seen for variants FT2 and BT6. Proteins belonging to the family of MFS transporters have been shown to contribute to tolerance to bile salt stress in *E. coli* (Paul *et al.*, 2014).

Interestingly, a trend was seen for new variants with an increased tolerance to both freeze-thaw and bile, as it appeared that variants derived from smaller-sized colonies had a higher tolerance to both types of stresses. This observation was further investigated in Paper III, where the growth kinetics of the new variants were compared with those of wild-type DSM 17938. By following the changes in optical density during cultivation under normal conditions, it was found that the two new variants derived from smaller-sized colonies indeed had changes in their growth kinetics. They had a longer lag phase compared with wild-type DSM 17938 (Figure 12), and also had a lower final optical density (data not shown). The variant derived from a large colony (BT5), on the other hand, had a growth curve which was similar to that of wild-type DSM 17938.

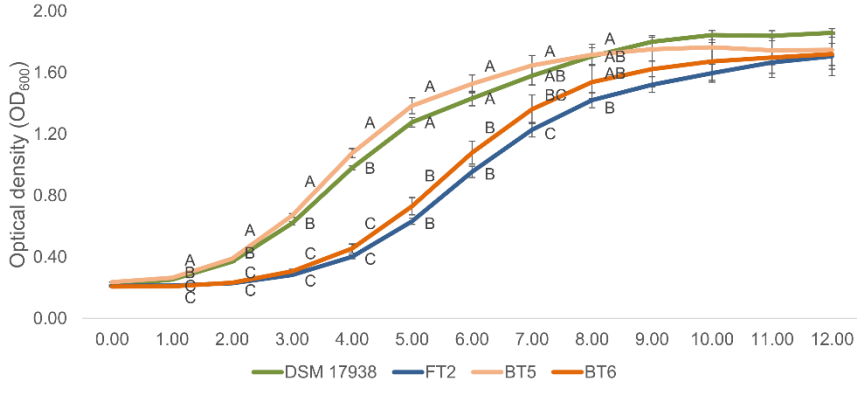

Figure 12. Growth curves (change in optical density measured at 600 nm (OD_{600})) of *Lactobacillus reuteri* strain DSM 17938 wild type and variants FT2, BT5 and BT6 during a 12-hour cultivation in de Man, Rogosa and Sharpe (MRS) broth at 37 °C. Mean ± SEM of six biological replicates from one experiment. Marker points with different letters are significantly different (p<0.05).

In Paper III, the observation made by Garcia *et al.* (unpublished) that higher freeze-thaw tolerance correlates to higher survival ability during lyophilisation was evaluated for the new variant FT2 compared with wild-type DSM 17938 (Table 3). One candidate from each of the other groups of variants was also tested, to investigate any changes in those. Most of the new variants did not significantly differ in survival ability compared with wild-type DSM 17938

(54.64%). However, the combination variant MFT3 had the highest survival ability (69.19%), significantly higher than all except variant BT6. Surprisingly, FT2 had the lowest survival ability (47.78%), which might indicate that the original hypothesis of a correlation between freeze-thaw tolerance and survival during lyophilisation is false. An earlier study suggested that this hypothesis might be true (Schoug, 2009), but other studies have reported a negative correlation, indicating that strains with higher freeze-thaw tolerance could have lower storage stability after lyophilisation (Sawicki, 2017).

Table 3. Viable counts (log_{10} CFU mL^{-1}) of *Lactobacillus reuteri* strain DSM 17938 wild type and variants FT2, MFT3, MB4 and BT6 after lyophilisation in a 10% sucrose solution. Mean values ± SEM of four replicates (two biological and two technical replicates). Values with different letters are significantly different ($p<0.05$)

Bacterial variant	Initial counts	After lyophilisation	% survival
WT	10.13 ± 0.01	9.87 ± 0.02	54.6^{B}
FT2	10.03 ± 0.02	9.71 ± 0.03	47.8^{B}
MFT3	9.97 ± 0.03	9.81 ± 0.02	69.2^{A}
MB4	10.12 ± 0.02	9.82 ± 0.02	51.1^{B}
BT6	10.04 ± 0.01	9.80 ± 0.03	57.9^{AB}

However, during the five-week stability test of each variant, FT2, BT6 and wild-type DSM 17938 had almost identical values for the viability counts. Variant MFT3 showed better stability for three weeks, but at the last time point (five weeks) its viable counts dropped sharply. In order to study whether the differences between the new variants and wild-type DSM 17938 were in activation time, rather than viable counts, the growth kinetics of the lyophilised cultures during normal cultivation conditions were analysed. The results showed similar results as seen in Figure 12, with longer lag phase for FT2 and BT6, but this time the final optical density was the same for all variants. According to the results in Paper III, variants with higher tolerance to repeated cycles of freeze-thaw are not necessarily more tolerant to the stressful conditions during the lyophilisation process.

In addition to maintaining viable counts at as high a level as possible when passing through the gastrointestinal tract, it is also important for probiotic bacteria to be able to stay in the small and large intestine long enough to exert an effect on the host. In order to remain in the intestine and also to come into as close as possible contact with the epithelium and/or the immune cells, the bacteria need to adhere, preferably to the mucus layer produced by the mucosal membranes of the intestine. Compared with other strains of reuteri, the mother strain of DSM 17938, named ATC 55730 or SD 2112 (Rosander *et al.*, 2008),

has been shown to have low mucus binding capacity (Saulnier *et al.*, 2011; Jonsson *et al.*, 2001), similar to that of DSM 17938 (Rosander *et al.*, 2008).

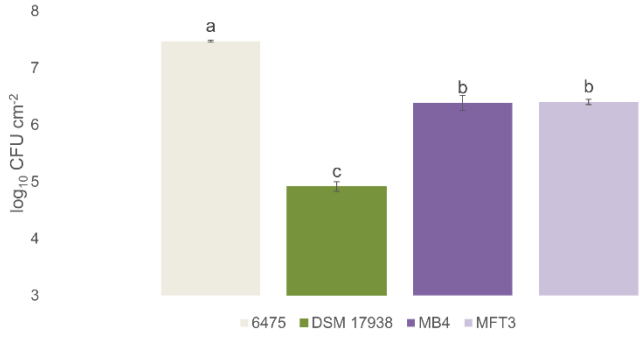

Figure 13. Mucus binding capacity of *Lactobacillus reuteri* strain DSM 17938 and new variants MB4 and MFT3 compared with strain ATCC PTA 6475, previously shown to be a good mucus binder. The capacity is shown as $\log_{10}$ CFU cm^{-2} (mean ±SEM). Bars with different letters are significantly different ($p<0.05$).

The results presented in Paper II showed that DSM 17938 indeed has a low mucus binding capacity *in vitro*, but that this could be improved 10-fold by the use of experimental evolution (Figure 13). Several variants of DSM 17938 were isolated during the mucus binding assay and saved. Two of those variants were used for further analyses. These were MB4, a 'pure' mucus binding variant, and MFT3, a mucus binding variant of the existing freeze-thaw variant FT2. The two new variants had a binding capacity of approximately 6.5 $\log_{10}$ CFU cm^{-2}, compared with 5 $\log_{10}$ CFU cm^{-2} for the wild type. An explanation for the increased mucus binding capacity of the new variants of DSM 17938 could not be found in the genomic data. However, proteomic analysis of variant MB4 revealed a few proteins which could possibly explain the new and improved phenotype. A protein similar to MsbA, an aminotransferase involved in lipid A and lipopolysaccharide transport in Gram-negative bacteria (Eckford & Sharom, 2010), was induced for MB4. MsbA has also been suggested to play a role in glycerophospholipid transport from the inner to the outer membrane of *E. coli* (Zhou *et al.*, 1998). Lipid A and LPS is not present in the cell wall of Gram-positive bacteria, but the presence of proteins similar to MsbA in *L. reuteri* indicate that it may also be involved in the transport of other lipids in lactobacilli. In addition to this protein, a LysM peptidoglycan-binding domain-containing protein was induced in MB4. This protein was similar in sequence to a previously described peptidoglycan endopeptidase present in *L. reuteri*. These enzymes are cell wall hydrolases, which have been show to correlate to increased

adhesion to hydrophobic surfaces for *Lactococcus lactis* and *Staphylococcus aureus* (Porayath *et al.*, 2018; Vollmer *et al.*, 2008; Mercier *et al.*, 2002).

Another plausible explanation for the increase in adhesion could be a change in other functional properties of DSM 17938. For variants MB4 and MFT3, an increase in hydrophobicity was seen compared with wild-type DSM 17938 (Paper III). A correlation between cell surface hydrophobicity and mucus adhesion has been demonstrated in a number of studies (Kos *et al.*, 2003; Ehrmann *et al.*, 2002; Del Re *et al.*, 2000; Wadstroum *et al.*, 1987b). When grown in MRS broth, another difference between MB4 and MFT3 compared with wild-type DSM 17938 was observed, in that overnight cultures of MB4 and MFT3, cultivated under normal conditions in either small glass tubes or large glass bottles, formed aggregates (Figure 14). Previous studies have observed that strains with higher mucus binding capacity also have significantly higher aggregation levels (Lukić *et al.*, 2012; MacKenzie *et al.*, 2010).

Figure 14. Aggregation seen in new variants of *Lactobacillus reuteri* strain DSM 17938 when cultivated in de Man, Rogosa and Sharpe (MRS) broth. (A) DSM 17938, (B and C) MB4 and (D) MFT3.

3.4 Evaluation of new DSM 17938 variants *in vitro* (Paper III)

In order to investigate whether other characteristics with possible connections to host-microbe interactions had been affected during the improvement process, DSM 17938 variants were evaluated using different *in vitro* models. Intestinal epithelial cell models were chosen to investigate whether the new variants with increased mucus binding capacity had increased binding capacity to host cells. The adhesion of *Lactobacillus* and other probiotic bacteria to intestinal epithelial cells is suggested to play a role in pathogen removal, by *e.g*. reducing the number of binding sites (Liu *et al.*, 2015; Roselli *et al.*, 2007). Wild-type DSM 17938 and the two variants with improved mucus binding capacity (MB4 and MFT3) were evaluated in both IPEC-J2 and Caco-2 cell models in Paper III. MB4 had similar adhesion to both cell lines as wild-type DSM 17938, while MFT3 had significantly lower binding capacity. According to these results, there is no correlation between mucus adhesion and epithelial cell adhesion, indicating different mechanisms for binding. One study has found a few proteins in *L. reuteri* that are involved in adhesion to mucus and to epithelial cells (Jensen *et al.*, 2014). However, since none of these proteins was found to be upregulated for MB4, and since only the mucus binding capacity was improved for this variant and not the adhesion to Caco-2 or IPEC-J2, the mechanism behind the adhesion is more likely due to other interactions. There are several other proteins with a possible role in adhesion of *Lactobacillus* to both mucus and epithelial cell lines, but none of these was found to be upregulated in the variants tested. There could instead be non-specific physical interactions behind the increased adhesion in MB4 and MFT3. Studies have shown correlations between adhesion to the small intestine of pigs (Wadstroum *et al.*, 1987a), porcine ileal epithelial cells (Kos *et al.*, 2003), human epithelial cell lines such as HT-29 (VIZOSO PINTO *et al.*, 2007) and cell surface hydrophobicity of the bacteria. A study on *Lactobacillus rhamnosus* GG has shown though, that reduced hydrophobicity of the strain has only a minor reducing effect on the adhesion to Caco-2 (Deepika *et al.*, 2009). This indicates that there may be a correlation between hydrophobicity and mucus binding capacity, but that it does not necessarily always correlate with epithelial cell adherence.

In intestinal permeability assays with IPEC-J2 cells, previous results have indicated that DSM 17938 has a protective effect against the increased permeability caused by ETEC (Karimi *et al.*, 2018). The experiments in Paper III confirmed those results, which were then compared with the results for the new variants. The results for the IPEC-J2 model revealed differences in the protective effect of some of the variants compared with the wild type (Figure 15). The mucus binding variant MB4 showed similar results to wild-type DSM

17938, while the bile tolerant (BT6) and freeze-thaw tolerant (FT2) variants showed no protective effects against ETEC. Pre-treatment with MFT3 did not give significant differences from the cells which only received the ETEC challenge, but there was a tendency for a protective effect. The lower protective effects of MFT3 compared with MB4 and wild-type DSM 17938 could possibly be explained by lower adhesion to IPEC-J2, since other studies have reported correlations between adhesion of ETEC and its effects on pro-inflammatory cytokine production (Kšonžeková *et al.*, 2016). In that study, lower adhesion of ETEC led to lower production of pro-inflammatory IL-1β and IL-6, after treatment with exopolysaccharides produced by DSM 17938.

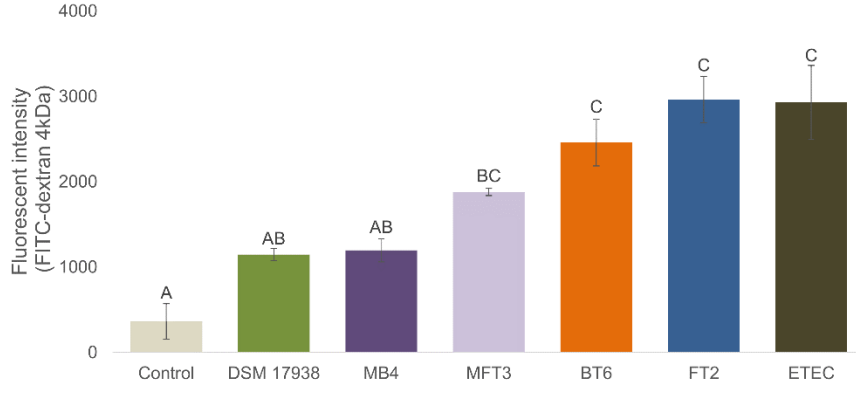

Figure 15. Results (mean ± SEM) showing leakage of fluorescent macromolecule FITC-dextran (4 kDa) during a permeability experiment using IPEC-J2 cells. Before challenge with ETEC, cells were either pre-treated with *Lactobacillus reuteri* strain DSM 17938 and new variants MB4, MFT3, BT6 and FT2, or not pre-treated (ETEC). Bars with different letters are significantly different (p<0.05).

The hypothesis that a variant of DSM 17938 with increased mucus binding capacity would also have higher adhesion to intestinal cells and a better protective effect against ETEC could not be proven in Paper III. While the mucus binding capacity was improved for MB4, the adhesion to cells was not. The protective effect against ETEC was not improved either for this variant, with results similar to those for DSM 17938. However, since IPEC-J2 is a cell line that does not produce any mucus (Schierack *et al.*, 2006), MB4 could have a better effect on mucus producing cell lines such as LS174T (MUC2) (Hatayama *et al.*, 2007). Administration of *L. reuteri* has been shown to lower the expression of pro-inflammatory cytokines both *in vitro* and *in vivo* (Karimi *et al.*, 2018; Bene *et al.*, 2017; Gao *et al.*, 2016; Zelante *et al.*, 2013). Since better adhesion

to mucus could allow a longer time to interact with immune cells, the mucus binding variants could be hypothesised to have a better inhibitory effect on the expression of pro-inflammatory cytokines.

The new variants were also evaluated for their effect in a cell model to study the possible effect on visceral pain, the TRPV1 model. The TRPV1 receptor has been shown to be upregulated in people with irritable bowel syndrome and is believed to be involved in visceral hypersensitivity (Akbar *et al.*, 2008). Since DSM 17938 has been shown to have a good effect against infantile colic, where one of the possible causes is believed to be painful intestinal contractions (Szajewska *et al.*, 2013; Savino *et al.*, 2010), the inhibition of the TRPV1 receptor might be one of the possible mechanisms behind this effect. Pre-treating Jurkat cells with DSM 17938 and the different variants (FT2, BT6, MB4 and MFT3) resulted in a reduction in activation of the TRPV1 receptors by capsaicin (Figure 16).

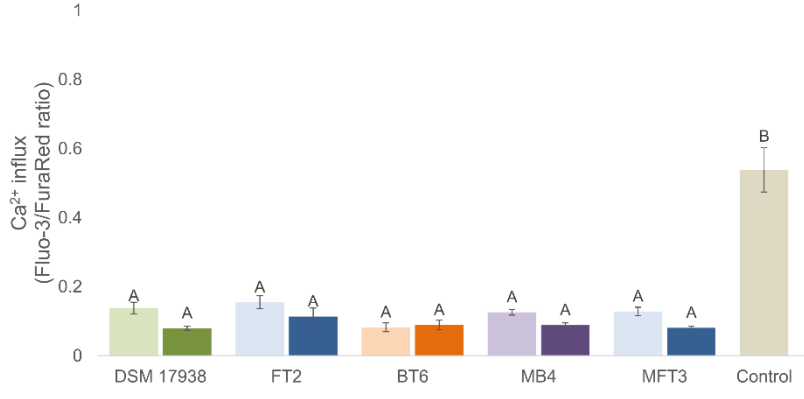

Figure 16. Results (mean ±SEM) from the visceral pain receptor model, using Jurkat cells expressing the TRPV1 receptor, showing Fluo-3/FuraRed ratios as values for calcium ion (Ca^{2+}) influx. Before challenge with capsaicin, cells were either pre-treated with *Lactobacillus reuteri* strain DSM 17938 and new variants FT2, BT6, MB4 and MFT3 with two ratios of bacteria/Jurkat cells (20:1 = light colour, 50:1 = dark colour) or not pre-treated (Control). Bars with different letters are significantly different ($p<0.05$).

Two ratios of bacteria to Jurkat cells were studied and both had an effect on the TRPV1 receptors, with a slightly larger decrease in Ca^{2+} influx in the ratio 50:1 compared with 20:1 for most variants. A previous study has suggested that DSM 17938 mediates its effect on TRPV1 receptors by secretion or shedding of products, since supernatants from cultures of DSM 17938 have similar effects as live bacteria, while dead bacteria have no effect (Perez-Burgos *et al.*, 2015). Those authors suggest that microvesicles produced by the bacteria might confer this effect. This would also explain how luminal bacteria could influence the enteric nervous system and have an effect *in vivo*, since microvesicles are small

enough to diffuse through the mucus layer and intestinal monolayer. To explain why no differences were seen between the new variants and wild-type DSM 17938, it might be necessary to analyse the production and content of microvesicles for each new variant compared with wild-type DSM 17398, which has been done for DSM 17938 in another study (Grande *et al.*, 2017).

4 Conclusions and future perspectives

As a probiotic, *L. reuteri* is one of the most clinically studied bacteria, with positive effects against a number of different diseases and disorders and the best documented effects seen against infantile colic. Infantile colic and a number of other gastrointestinal diseases are suggested to be connected to a leaky gut (increased intestinal permeability). Both *in vitro* and *in vivo* studies using animal and cell models have shown that this increase in permeability can be reduced or even normalised by the use of probiotic bacteria and compounds produced by these bacteria.

Paper I investigated whether cultivation of *L. reuteri* in a cereal-based substrate could stimulate the bacteria into producing compounds with possible biological activity, *i.e.* production of bioactive compounds. Fermentation of barley varieties with different characteristics, with strains of *L. reuteri* of different origin were made. The impact of nutrient content and strain properties as well as ecological background on the bacterial densities and metabolic processes were evaluated. The results revealed some degree of strain adaptation to a specific niche in *L. reuteri*, but the differences between the strains were relatively small, both in the effect on bacterial densities and on metabolic profiles. The impact of the substrate was great for some strains, but not for others. Correlations between the presence of higher levels of tryptophan and increased growth were seen for DSM 17938 and to some degree for the other strains. The supernatants from these combinations were shown to contain different levels of tryptophan catabolites, which have previously been shown to be bioactive compounds with effects on innate immune responses in animal models. Further experiments are needed to confirm production of those catabolites in barley fermented by *L. reuteri*, as only increased concentrations of these catabolites could be observed. Tests on the effect of possible bioactive compounds produced during the fermentation of barley revealed only minor protective effects on the permeability induced by ETEC after pre-treatment with some supernatants from barley flour fermented by *L. reuteri*. No correlations to

a specific content of bioactive compounds were found. Optimisation of production of such bioactive compounds may be needed, since the levels might have been too low to exert an effect in the models.

The majority of clinical studies investigating the probiotic effect of *L. reuteri* have been performed on strain DSM 17938. While it has good clinical effects, there are certain functional properties of the strain which, if improved, might improve the host-microbe interactions. Bile tolerance and mucus binding capacity are two properties for which some other strains of *L. reuteri* outperform DSM 17938. In Paper II, experimental evolution was used as a tool to isolate new variants of DSM 17938 with improved tolerance to porcine bile and with higher adhesion to porcine mucus. Another more technical property (freeze-thaw tolerance) was also improved for variants of DSM 17938. Combined tolerance to freeze-thaw stress and increased mucus binding was found in one variant. The new variants were shown to have no or only few changes in the genome sequence, indicating a low mutation rate. Analyses of the proteome, on the other hand, revealed changes for all variants compared with the wild type, with the bile tolerant and freeze-thaw tolerant variants being very similar in protein expression, while the mucus binding variant had changes in other proteins. The changes in the bile and freeze-thaw tolerant variants were mainly suppression of an operon of genes involved in type II fatty acid synthesis and the induction of a MFS transporter protein possibly involved in increased bile tolerance. For the mucus binding variant, the analysis revealed induction of a protein with a possible involvement in glycerophospholipid transport in Gram-negative bacteria and proteins involved in cell wall hydrolysis that is involved in adhesion to hydrophobic surfaces in other lactic acid bacteria. No changes in proteins known to be involved in mucus binding were seen, and use of an alternative technique, such as bacterial surface shaving followed by analysis of the released proteins, might be needed to identify more changes in the membrane and cell wall proteome. Repeating the initial experiments with knock-out mutants of DSM 17938 with inactivations of the genes believed to be involved in stress tolerance and mucus binding may also be needed, to confirm the assumptions made in Paper II.

In Paper III, the new variants with improved functional properties were studied in different *in vitro* models, to determine possible changes in other characteristics and to study the effect of the variants on intestinal permeability and pain receptor TRPV1 compared with wild-type DSM 17938. A possible connection between freeze-thaw tolerance and ability to survive lyophilisation was tested, but no clear correlations were seen. A possible connection between colony size and tolerance to bile and freeze-thaw was detected during the initial experiments in Paper II. Variants derived from small colonies generally had a

higher tolerance to both bile and freeze-thaw stress than variants derived from normal-sized colonies. The hypothesis that better stress tolerance could come at the cost of disturbed growth was confirmed in Paper III, which showed a longer lag phase and lower final optical density of variants derived from small colonies. Previously reported correlations between higher hydrophobicity and increased mucus binding were confirmed for the mucus binding variants, but no increased binding to intestinal epithelial cells was seen for the variants compared with wild-type DSM 17938. The protective effect of wild-type DSM 17938 against increased intestinal permeability was also seen in one of the mucus binding variants, but was significantly lower in the bile and freeze-thaw tolerant variants. The differences in protective effects for some variants could partly be explained by differences in adhesion to the intestinal epithelial cells. In contrast, there were no differences between the new variants compared with wild-type DSM 17938 in the inhibitory effect on pain receptor TRPV1.

In future studies, the importance of the new properties in actual colonisation of the gastrointestinal tract of both animals and humans should be evaluated for the variants produced in this thesis, by studying biopsies from different parts of the intestine for the presence of *L. reuteri*. Previous studies have successfully studied colonisation by ATCC 55730 using 16S molecular beacon probes shown to be species-specific (Valeur *et al.*, 2004). A less invasive way could be to determine the effect of colonisation of the new variants, and not actual colonisation. This could be done by repeating earlier animal and human studies, where increased mucus binding capacity could be hypothesised to play an important role, *e.g.* in studies on inflammatory intestinal diseases where immune responses (*e.g.* expression of pro-inflammatory cytokines) play a crucial role (Neurath, 2014).

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Popular science summary

Probiotics are live microorganisms which, when eaten in sufficient quantities, give a positive health effect. Most of the probiotic products on the market contain lactic acid bacteria. *Lactobacillus reuteri* is a species belonging to this group of bacteria. It occurs naturally in the intestine of birds and mammals. In humans, it is often found in the mouth, gastrointestinal tract, breast milk and the vagina. It was once prevalent in the gut of most humans, but because of lifestyle changes it has become rarer. Nowadays, it can be found in a range of different probiotic products and is one of the most clinically studied probiotic bacteria in the world. As a probiotic supplement, it has been shown to help against a number of different diseases and disorders. The best effects have been seen in infantile colic, but it has also been shown to alleviate diarrhoea and intestinal pain in both children and adults. As the name indicates, the lactic acid bacteria produce lactic acid, but they also produce a range of other compounds with biological activity and hence have a positive health effect. These include vitamins and other antioxidants, which can also be found in fruits, vegetables and whole grain products, but also compounds produced by the microorganisms in the human gastrointestinal tract.

The starting point for *L. reuteri* as a probiotic bacteria was when scientists in the 1980s showed that it could produce an antimicrobial compound, which was given the name reuterin. Since then, studies have shown that it can produce a number of other biologically active compounds. When *L. reuteri* is used to ferment whole grain flour, it can produce *e.g.* antioxidants (polyphenols) and antimicrobial substances. Cereals are a good substrate for cultivating *L. reuteri*, and sourdoughs are actually the only environment outside the body of animals and humans where it has been isolated. The first part of this thesis investigated whether different strains of *L. reuteri* produce bioactive compounds during fermentation of whole grain barley flour. The results showed that the fermented barley contained a number of different biologically active compounds, *e.g.* gamma-aminobutyric acid (GABA), which is a signal substance in the central

nervous system, but also indole-3-aldehyde, serotonin and tryptamine, which are derived from the essential amino acid tryptophan and are all highly interesting bioactive compounds. Indole-3-aldehyde and tryptamine activate specific receptors (aryl hydrocarbon receptors, AhR) which are involved in the innate immune response. Serotonin and GABA are involved in the central nervous system. These compounds were already present in the flour before the fermentation, but the amount was increased during fermentation by *L. reuteri*.

In order for probiotic bacteria such as *L. reuteri* to have a positive health effect through *e.g*. the production of bioactive compounds, they need to survive different kinds of stressful situations. First of all, they need to survive the industrial production process and have a long shelf-life. Bacteria used in probiotic products are often freeze-dried so that they can be stored during a long period in a sort of hibernation state, before coming to life following consumption. Freeze-drying is a tough process and up to half of the bacterial cells can die even when the process is optimised. In an attempt to increase the survival ability of a *L. reuteri* strain called DSM 17938 (Protectis) during and after freeze-drying, the bacteria were subjected to repeated cycles of freezing and thawing. By isolating bacteria that survived, variants that could tolerate the freeze-thaw stress up to 10 times better than the original strain were found. However, there were no indications that the new variants could tolerate freeze-drying better than the original strain.

Having survived the stress during industrial production, the bacteria then need to survive the harsh environment of the gastrointestinal tract. Strain DSM 17938 has been shown to tolerate the acidic conditions of the stomach, but it is more sensitive to bile than other strains of *L. reuteri*. The function of bile is to dissolve fats in the diet, but it also has an inhibitory effect on many bacteria. To protect themselves, certain bacteria have developed strategies involving *e.g*. breaking down the bile, covering themselves in a protective layer or pumping the bile out from the cells. By exposing the bacteria to bile, new variants with almost 1000-fold better tolerance to bile were isolated.

A third property considered important for probiotic bacteria is binding to the mucus layer within the small and large intestine. The mucus layer acts as a physical barrier to prevent harmful bacteria from coming into contact with the intestinal cells and going on to enter the body. To remain in place long enough to communicate with *e.g*. immune cells, and thereby have a health-promoting effect, probiotic bacteria need to be able to bind to the mucus layer. While DSM 17938 is an effective probiotic bacterium, it is relatively bad at binding to intestinal mucus. Using a method for selecting bacteria with a higher binding capacity, new variants with a 10-fold increase in mucus binding capacity were isolated.

In order to find out whether bacteria are good probiotic candidates, they first need to be evaluated using different kinds of cell models before being tested in clinical studies. Strain DSM 17938 has previously been shown to have a good effect in a number of different cell models. These include a model with intestinal epithelial cells which are used to simulate a leaky gut, but also a model with cells that express receptors involved in pain perception in the intestine. The new variants of DSM 17938 were tested in these two models and the inhibitory effect on increased intestinal leakage and increased activation of the pain receptors was compared with that of the original strain. All variants tested had a similar effect on activation of the pain receptors, but some seemed to have lost the protective effect against a leaky gut.

In conclusion, a number of variants of *L. reuteri* DSM 17938 with increased stress tolerance and mucus binding capacity were successfully isolated. To better understand how these improved properties can affect the probiotic effect, further evaluations in more complex models and in clinical studies are needed. However, the variants of DSM 17938 isolated in this thesis can be a good help in understanding what is required of a bacteria to have a good health effect.

Populärvetenskaplig sammanfattning

Probiotika är levande mikroorganismer som när man äter dem i tillräcklig mängd ger en positiv effekt på hälsan. De flesta probiotiska produkterna som finns på marknaden innehåller mjölksyrabakterier och *Lactobacillus reuteri* är en art som tillhör denna grupp av bakterier. Arten finns naturligt i tarmen hos både fåglar och däggdjur. Hos människor finns den ofta i munnen, mag- och tarmsystemet, bröstmjölk samt i vaginan. Tidigare förekom den troligtvis hos nästan alla människor, men på grund av en ändrad livsstil har den blivit allt mer sällsynt. Numera finns den dock i en rad probiotiska produkter och är en av de mest kliniskt studerade probiotiska bakterierna i världen. Som ett probiotiskt tillskott har *L. reuteri* visat sig kunna minska problemen med olika sjukdomar och funktionella störningar. De bästa effekterna har man sett på spädbarn med kolik, men den har även visat sig kunna lindra diarré och magsmärta på både barn och vuxna.

Som namnet mjölksyrabakterier indikerar bildar dessa bakterier mjölksyra, men dessutom bildar de en rad andra ämnen som har en biologisk aktivitet och därmed en positiv påverkan på hälsan. Det kan till exempel vara vitaminer och antioxidanter som finns i frukt, grönsaker och fullkornsprodukter, men det kan också vara ämnen som produceras av mikroorganismer i vårt tarmsystem. Startskottet för användandet av *L. reuteri* som probiotisk bakterie var när forskare på 1980-talet visade att den kunde producera en antimikrobiell substans som gavs namnet reuterin. Sedan dess har studier visat att den kan bilda en rad andra biologiskt aktiva ämnen. När bakterien fermenterar fullkornsmjöl har man sett att den kan bilda bl.a. antioxidanter (polyfenoler) och antimikrobiella substanser. Spannmål fungerar bra för att odla *L. reuteri*, och surdeg är faktiskt den enda miljön utanför kroppen på djur och människor som bakterien har isolerats från. I den första delen av denna avhandling undersöktes om olika stammar av *L. reuteri* bildar biologiskt aktiva ämnen vid fermentering av fullkornsmjöl av korn. Det visade sig att fermenterat korn innehöll en rad olika biologiskt aktiva ämnen, bl.a. gammaaminosmörsyra (GABA) som är en

signalsubstans i det centrala nervsystemet, men också indol-3-aldehyd, serotonin och tryptamin som bildas från aminosyran tryptofan och som alla är mycket intressanta biologiskt aktiva ämnen. Indol-3-aldehyd och tryptamin aktiverar speciella receptorer (AhR, aryl hydrocarbon receptor) som är inblandade i aktivering av det ospecifika immunförsvaret. Serotonin, likt GABA, är även den involverad i det centrala nervsystemet. Ämnena kunde hittas i mjölet innan fermenteringen, men mängden av dem ökade vid fermentering med *L. reuteri*.

För att probiotiska bakterier som reuteri ska kunna ha en positiv effekt på hälsan genom bl.a. bildandet av biologiskt aktiva ämnen, måste de överleva olika typer av stress. De måste först och främst överleva den industriella produktionen och helst ha en så lång hållbarhet som möjligt. Bakterier som används i probiotiska produkter är ofta frystorkade för att kunna förvaras under en lång tid i ett slags vilande tillstånd, och sedan väckas upp när de konsumeras. Frystorkning är en tuff process och även om den är optimerad kan uppåt hälften av bakterierna dö. I ett försök att förbättra överlevnaden vid och efter frystorkning hos en stam av *L. reuteri* kallad DSM 17938 eller Protectis, utsattes bakterien för upprepade infrysningar och efterföljande tining. Genom att isolera de bakterier som överlevde hittades bakterier som tålde frys-tina-stressen upp till tio gånger bättre än ursprungsstammen. Dock kunde man inte se att den nya varianten kunde klara frystorkning bättre än den ursprungliga stammen.

Men det är inte bara viktigt att den ska överleva stressen vid den industriella produktionen utan bakterien måste även överleva i den hårda miljön i mag- och tarmkanalen. DSM 17938 har visat sig tåla de sura förhållandena i magsäcken bra, men studier har visat att den i vissa fall är mer känslig för galla jämfört med andra stammar av *L. reuteri*. Galla har som funktion att lösa upp fett i vår kost men har även en hämmande effekt på många bakterier. För att skydda sig har vissa bakterier utvecklat strategier för att bryta ner gallan, täcka sig med ett skyddande lager, eller aktivt pumpa ut gallan från cellen. Genom att utsätta bakterien för galla kunde nya varianter med nästan 1000 gånger bättre tålighet mot galla isoleras. En tredje egenskap hos probiotiska bakterier som anses vara viktig, är inbindning till slemhinnorna i tunn- och tjocktarm. Dessa slemhinnor agerar som en fysisk barriär för att förhindra skadliga bakterier från att nå tarmcellerna och ta sig vidare in i kroppen. För att stanna kvar tillräckligt länge och kommunicera med bl.a. immunceller och därmed ha en effekt på hälsan, måste de probiotiska bakterierna kunna binda till slemhinnorna. Trots att DSM 17938 är en effektiv probiotisk bakterie är den relativt dålig på att binda till slemhinnor. Med en metod för att selektera fram bakterier med högre bindande förmåga kunde nya varianter med tio gånger högre bindningsförmåga isoleras.

För att identifiera lovande probiotiska kandidater, brukar de först utvärderas m.h.a. olika typer av cellmodeller, innan de testas i kliniska försök. DSM 17938

har tidigare visat sig ha bra effekt i en rad olika typer av cellmodeller, bl.a. i en modell med tarmepitelceller där en läckande tarm simuleras, men också i en modell med celler som uttrycker receptorer som är involverade i smärtupplevelsen i tarmen. De nya varianterna av DSM 17938 testades i dessa två modeller och den inhiberande effekten på ökande läckage och ökad aktivering av smärtreceptorer jämfördes med den ursprungliga stammen. Samtliga varianter som testades hade liknande inhiberande effekt på aktivering av smärtreceptorerna. Dock verkade vissa varianter ha tappat den skyddande effekten mot en läckande tarm. Sammanfattningsvis så har en rad varianter av *L. reuteri* DSM 17938 med ökad förmåga att tåla stress och binda till slemhinnor tagits fram, men för att bättre förstå hur de förbättrade egenskaperna påverkar den probiotiska effekten krävs ytterligare utvärdering i mer avancerade modeller samt i kliniska studier. De framtagna varianterna av DSM 17938 kan även vara till god hjälp för att bättre förstå vad som krävs av en bakterie för att ha en bra effekt på hälsan.

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