# Fermentation of Barley Flour with *Lactobacillus reuteri*

A source of bioactive compounds against a leaky gut?

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Licentiate Thesis
Swedish University of Agricultural Sciences
Uppsala 2015

Cover: Photos of cereal flour fermented with *L. reuteri*, colonies of *L. reuteri* on MRS agar, a 1H-NMR spectrum and a light microscopy image of IPEC-J2 cells (photo: Anton Pallin)

ISBN (print version) 978-91-576-9282-5
ISBN (electronic version) 978-91-576-9283-2

Print: SLU Service/Repro, Uppsala 2015

# Fermentation of Barley Flour with *Lactobacillus reuteri*. A source of bioactive compounds against a leaky gut?


## Abstract

Fermentation of foodstuffs has beneficial effects on shelf life, taste and texture and possibly also health of the consumer. Products containing microbes with beneficial health effects for the host are defined as probiotics. One probiotic bacterium, *Lactobacillus reuteri*, has been shown to exert positive effects on a number of diseases and disorders, including a possible protective role against increased intestinal permeability or 'leaky gut'. Increased intestinal permeability has been linked to *e.g.* inflammatory bowel disease (IBD), irritable bowel disease (IBS), coeliac disease and infectious diarrhoea. This study examined the effect of different cereal substrates on the growth, general metabolism and production of potential bioactive compounds by *L. reuteri*, with the aim of establishing a synbiotic formulation effective against increased intestinal permeability. Combinations of six barley varieties and six strains of bacteria were evaluated using standard plate counts, chemical analysis ($_1$H-NMR) and a small intestinal epithelial cell model (IPEC-J2). The overall growth of *L. reuteri* in barley flour was good and reached higher densities in untreated compared with heat-treated flours. Differences in growth were also seen between bacterial strains and barley varieties. The general metabolism was similar for all strains with a few exceptions, *e.g.* lower production of succinate from *L. reuteri* DSM 17938. Two potentially bioactive compounds, γ-aminobutyric acid (GABA) and 3-hydroxypropionic acid (reuterin), were detected in barley fermented by *L. reuteri*. GABA was present in unfermented flour at similar levels as in the fermented counterpart, indicating no or low production by *L. reuteri*. Reuterin production was detected as formation of 1,3-propanediol and was strain-specific, being present in fermentation with DSM 17938 and ATCC PTA 6475. Experiments with IPEC-J2 cells revealed an increase in epithelial permeability caused by untreated flour, both fermented and unfermented. Treatment with heat-treated flour had a slight increasing effect on permeability, but recovered over time. Pre-treatments with live bacteria or fermented heat-treated flour before challenge with enterotoxigenic *E. coli* revealed significantly lower leakage of a molecular probe (FITC-dextran, 4 kDa). However, neither live bacteria nor their metabolites had a protective effect on epithelial permeability, measured as transepithelial electrical resistance (TEER).



*Keywords: Lactobacillus reuteri*, probiotics, prebiotics, synbiotics, intestinal barrier integrity, NMR, barley, IPEC-J2, cereal fermentation



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*Do… or do not. There is no try.*

Master Yoda

# 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 and Stefan Roos. (2015) Evaluation of growth, metabolism and production of potentially bioactive components during fermentation of barley with *Lactobacillus reuteri*. *Manuscript*.

II Anton Pallin, Torbjörn Lundh, Hans Jonsson and Stefan Roos. (2015) Metabolites produced by *Lactobacillus reuteri* during cereal fermentation prevent increased intestinal permeability caused by ETEC *in vitro*. *Manuscript.*

The contribution of Anton Pallin to Papers I and II was as follows:

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

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

# Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| CFU | Colony-forming units |
| ETEC | Enterotoxigenic *Escherichia coli* |
| FITC | Fluorescein isothiocyanate |
| GABA | Gamma-aminobutyric acid |
| GBF | Germinated barley foodstuff |
| GI | Gastrointestinal |
| HDL | High density lipoprotein |
| IBD | Inflammatory bowel disease |
| IBS | Irritable bowel disease |
| LAB | Lactic acid bacteria |
| LDL | Low density lipoprotein |
| MLSA | Multi-locus sequence analysis |
| NMR | Nuclear magnetic resonance |
| PCR | Polymerase chain reaction |
| SCFA | Short-chain fatty acids |
| TEER | Transepithelial electrical resistance |
| TJ | Tight junction |

# 1 Introduction

## 1.1 History of fermentations

Fermentation is considered the second oldest method for preserving food in the world, after drying (Farnworth, 2008). It is the process of transforming simple raw materials into different products with added value by exploiting the growth and activity of microorganisms on different substrates. People soon found other advantages of fermentation, *e.g*. not only could they store their food for a longer time, but they could also change the taste, texture and overall sensory sensation of that food. Other advantages regarding health and nutritional benefits also emerged. Wine making allowed people to have a beverage which would not cause sickness, since water supplies in larger settlements could be contaminated. In bread making, fermenting the flour-water mixture before baking led to a product with better sensory qualities and more easily available nutrients. The history of fermented foods is thought to have begun with cheese and bread making as long as 8000 years ago, in present-day Iraq (Fox, 1993). Documentation of such early fermentation comes from writings, drawings and friezes. Deliberate fermentation is believed to have been used as soon as people started domesticating cows, sheep, goats *etc*. Even though they did not know exactly what happened, people realised that they could keep milk for longer if they stored it in animal stomachs. Being stored in stomachs curdled the milk and exposed it to lactic acid bacteria and other microbes present in the environment, which formed the first primitive cheeses. An advantage of fermented milk and other food items was that they did not have to be further processed, *e.g*. by heating or cooking. As soon as they had been fermented, they were ready to be consumed (Hui *et al.*, 2003). For many thousands of years, people used the art of fermenting to produce different varieties of food items in order to extend the time for which they could store them or to achieve specific aromas or textures, without really knowing anything about the science

behind this. It would take until the mid-nineteenth century before an understanding of how the process works was reached, which changed the way in which food fermentation was performed. Two events in particular are considered to have been important during the nineteenth century. First, the Industrial Revolution changed the way in which food was produced. With growing populations in cities and towns, foods had to be made in larger quantities at an industrial scale, which changed the manufacturing process and demanded better knowledge of the process. At around the same time, the field of microbiology began to evolve as a science, which helped understand the biology behind fermentation and contributed to a more targeted and controlled approach to fermentation of foodstuffs (Caplice & Fitzgerald, 1999).

Cereal grain has long been the most important source of dietary proteins, carbohydrates, vitamins, minerals and fibre for people across the planet. However, a few different attributes cause cereal grain to have slightly lower nutritional quality than milk and milk products. For example, it usually has a lower protein content, lacks certain essential amino acids, has low starch availability and may also have anti-nutrients present (phytic acid, tannins and polyphenols). These anti-nutrients can form complexes with minerals (iron, zinc, calcium and magnesium) and proteins, lowering uptake of these in the gastrointestinal (GI) tract (Chavan *et al.*, 1989).

In order to increase the nutritional value of cereal grain, a number of different methods have been employed, ranging from plant breeding and supplementation of amino acids to processing technologies. The process technologies used to date include cooking, sprouting, milling and fermentation of the grain, of which fermentation is considered to be the most effective in improving the nutritional value (Blandino *et al.*, 2003). The processing of cereals by natural fermentation leads to the nutritional value being improved in a number of ways. For example, the levels of non-digestible polysaccharides and oligosaccharides are decreased, while amino acids and B-group vitamins are synthesised or increase in availability (KAZANAS & Fields, 1981). The pH of the fermented cereal is also decreased, which improves the enzymatic degradation of phytate by plant or microbial phytases, in turn increasing the amount of soluble minerals such as iron, zinc and calcium (Lioger *et al.*, 2007; Leenhardt *et al.*, 2005; Lopez *et al.*, 2001).

In addition to being able to affect the nutritional value of cereals by fermentation, fermentation microorganisms also improve the shelf life and sensory attributes (including texture, taste and aroma) of the final product (Figure 1). Sourdough and bread produced using specific strains of lactic acid bacteria (LAB) have been shown to retard the growth of moulds responsible for the spoilage of breads (Dal Bello *et al.*, 2007). Fermenting cereals with

bacteria has also been shown to increase the concentrations of volatile compounds such as the aromatics ethyl acetate and diacetyl, which can impart a more appealing smell and taste to the final product (Damiani *et al.*, 1996; Hansen & Hansen, 1994). The microbial community of fermented products can be quite complex and in some naturally fermented products the microbiology is mostly unknown. Fermented products usually contain a mixed culture of different bacteria, yeasts and moulds, some of which participate during the entire process while others only take part during some of the fermentation period due to changes in the dominant microbiota. The most used and most isolated bacteria found in fermentation include species of *Bacillus*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Pediococcus* and *Streptococcus* (Blandino *et al.*, 2003).


*Figure 1.* A selection of different fermented food items: sourdough, sour milk, red wine and cheese. Combined pictures from Wikimedia Commons, credited to Janus Sandsgaard, Kristofer2, André Karwath & Myrabella.

## 1.2 Barley (*Hordeum vulgare*)

Barley (*Hordeum vulgare*), like all other true cereals, is a member of the grass family. Barley, wheat, maize, rice, rye, millet, oats, sorghum and triticale are the nine most important cereals grown in the world today. Globally, barley is the fourth most produced cereal, after maize, rice and wheat (FAOSTAT, 2007). Barley is grown in different environments ranging from sub-Artic to sub-tropical and is mainly produced in temperate areas and at high altitudes of the tropics and subtropics. At lower altitudes of tropical areas barley is rarely grown, since it is not well-suited to warm, humid climates (Nevo & Shewry, 1992). The origins of barley have been traced back almost 10 000 years to an area in the Middle East known as the Fertile Crescent (Badr *et al.*, 2000). Early use of barley as a food dates back to the ancient Greeks, who made unleavened bread from barley flour. Even the Romans used barley, since they thought it a hearty tasting, high-energy food which would give their gladiators strength and stamina; the gladiators were actually called '*hordearii*' or 'barley men' (Baik & Ullrich, 2008). During the Bronze Age (2200 years ago), barley was one of the major cereals grown and used for food in Scandinavia. The main reason why barley was cultivated in these countries was that the wheat cultivars in those days were not suitable for growing in cold climates. With the discovery of new cereals and new cultivars and the method for leavening bread by addition of microorganisms, the use of barley in bread making was reduced. The probable reason for this reduction in barley use is that other cereals such as wheat gave a more light and airy bread that was probably more appealing.

Today, barley is used in breakfast cereals, stews, soups, porridge, bakery flour blends and baby food. In Korea, it is also used as a substitute for rice and for the production of soy paste and soy sauce. Most production of barley takes place in Europe (62%), followed by Asia (15%) and America (13%). The use of barley in food is very low, however. In the United States, only 1.5% of the barley produced is used for food and the rest is used for animal feed (65%) and in malt and alcohol production (30%). Figures from 1991 show that the use of barley for food in 12 countries in Europe averaged around 0.3%, while the use in *e.g.* China, India and Ethiopia was above 60% (Newman & Newman, 2006). While the present use of barley in food is low, an increase could be expected due to increasing health trends and an increasing number of reports on the possible health effects of consuming barley products

Barley has a high nutritional value, with low fat content, complex carbohydrates and a protein content that covers most of the amino acid requirements in humans. Barley grains also include minerals, vitamins (vitamin E in particular), other antioxidants, *e.g.* polyphenolic compounds, and insoluble and soluble fibre (Baik & Ullrich, 2008). Barley and oats are known

as high-fibre cereals, with relatively high levels of β-glucan compared with other cereals. β-glucan is a soluble fibre mainly found in the endosperm wall of cereals (Figure 2). In barley, it constitutes 75% of the endosperm cell wall (Fincher, 1975) and 2-10% of the total weight (Henry, 1987). Several studies have examined the health effects of barley β-glucans, with lowered blood cholesterol and glycaemic index being two promising results reported in clinical trials (Pins *et al.*, 2007). Barley β-glucan has also been shown to have an effect on weight loss by increasing satiety and by speeding up the passage of food in the colon (Schroeder *et al.*, 2009; Granfeldt *et al.*, 1994). All of these effects can be associated with a lower risk of heart disease and type-2 diabetes, showing the potential health effects of supplementing a diet with whole-grain barley products.

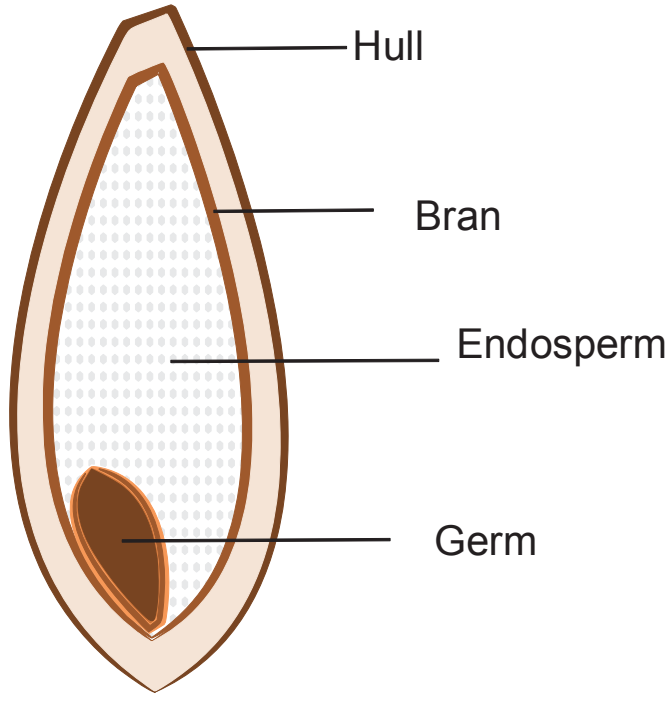


*Figure 2.* Figure showing a cross-section of a barley kernel with major components (hull, bran, endosperm and germ).

In addition to the positive effects on blood cholesterol and glycaemic index, barley has also been evaluated as a prebiotic with promising results when treating patients with ulcerative colitis (Kanauchi *et al.*, 2002). Both clinical activity and mucosal inflammation were reduced in patients with ulcerative

colitis given a daily supplementation of germinated barley foodstuff (GBF). Increased luminal butyrate production and stimulated growth of protective bacteria were suggested as the possible mechanism behind the decrease in inflammation. GBF has also shown promising results as an anti-tumour agent against colon cancer in a rat model (Kanauchi *et al.*, 2008). Differences seen in the GBF group compared with the control were a significant increase in the production of a tumour suppressor gene (slc5a8) and in caecal butyrate content. Studies with weaned piglets have also shown that a feed with different combinations of hulless and hulled barley supplemented with β-glucan could have a positive effect on the lactobacilli counts in the intestine (Pieper *et al.*, 2008). All these results suggest that the carbohydrate composition of a feed or a food item could have an effect on the microbial community structure in the intestine, or at least on a few specific species or genera of bacteria.

## 1.3 Prebiotics

The term 'prebiotic' refers to "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995). In principle, all prebiotics are fibres, but that does not necessarily mean that all fibres are prebiotics, since in order to be classified as a prebiotic a fibre has to fulfil a number of specific requirements. The food ingredient (or fibre) needs to be able to resist gastric acidity, hydrolysis by mammalian enzymes and absorption in the upper part of the gastrointestinal tract. Since the main purpose of the prebiotic is to stimulate the activity and/or growth of intestinal bacteria with a positive effect on human health, the last requirement of a prebiotic is that it should be fermentable by the intestinal microbiota. Food ingredients classified as prebiotics include non-digestible oligosaccharides in general and fructooligosaccharides in particular. Oligosaccharides that have been shown to have a prebiotic affect include inulin, lactulose and transgalacto-oligosaccharides (Gibson *et al.*, 2004). Prebiotics are usually designed to target bacteria of the genera *Bifidobacterium* and *Lactobacillus*, but effects on growth and activity are more common for bifidobacteria, since they seem to have a preference for oligosaccharides.

There are a number of different health effects related to the consumption of prebiotics. Some of these health effects can be correlated to the production of different short-chain fatty acids (SCFA), one of which is butyrate. Butyrate is the principal fuel for colonic epithelial cells and has been shown to stimulate apoptosis in the intestine, and may hence have a protective role against carcinogenesis (Sengupta *et al.*, 2006). Prebiotics have been shown to decrease inflammation by stimulating the growth of lactobacilli and bifidobacteria and the production of *e.g.* lactic acid (Rumi *et al.*, 2004). The production of lactic acid is considered to be able to lower the luminal pH and hence reduce the number of pathogenic bacteria. There are also some indications that prebiotics might be able to prevent the development of allergies in infants by alteration of the microbiota (Moro *et al.*, 2006). However meta-analyses of studies examining the effect of prebiotics in preventing allergic symptoms have found that the quality of the effect is very low (Osborn & Sinn, 2013). Infants have also been shown to be less afflicted with diarrhoea, flatulence, vomiting and fever when administered a daily supplement of oligofructose, compared with a placebo group, and have higher numbers of bifidobacteria and lower numbers of clostridia (Waligora-Dupriet *et al.*, 2005). A meta-analysis performed on

studies on obese or overweight adult patients has shown that prebiotics can reduce total plasma cholesterol and low density lipoprotein (LDL) concentrations and increase high density lipoprotein (HDL) concentrations in diabetic patients with overweight or obesity (Beserra *et al.*). Thus some evidence of potential health benefits of prebiotics exist, but further studies are needed to confirm many of these suggested effects. Barley contains a number of different compounds which have been shown to exhibit prebiotic effects, *e.g*. arabinoxylan, β-glucan and fructan (Gullón *et al.*, 2014; Peshev & Van den Ende, 2014; Mitsou *et al.*, 2010). This makes barley interesting as a source for prebiotics or for use in a synbiotic formulation.

## 1.4 Probiotics

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit to the host. The term probiotic is a composite of the Latin word '*pro*' meaning 'for' and the Greek word '*biotic*' or '*bios*' meaning 'life', and was first used to describe growth-promoting factors produced by microorganisms (Lilly & Stillwell, 1965). However, it was Metchnikoff who in 1907 became the first scientist to claim that consuming microorganisms could improve people's health. He claimed that lactobacilli in yoghurt could outcompete toxin-producing bacteria in the gut and thereby increase the human lifespan (Schrezenmeir & de Vrese, 2001). At around the same time, a French paediatrician named Henry Tissier observed that faecal samples taken from children with diarrhoea contained lower amounts of bacteria with a strange Y-shaped morphology. These bacteria were later named *Bifidobacterium* and, according to Tissier's observations, they were far more abundant in healthy children, so he suggested administering these bacteria in order to restore the microbiota in the gut (Tissier, 1907). When choosing a microbe for use as a probiotic, there are a number of different attributes or factors that are important or necessary. Resistance to pancreatic enzymes, acid and bile are important in order for the probiotic microbe to survive the passage through the GI tract. Adhesion to the intestinal mucosa might also be important for persistence, immunomodulation, pathogen exclusion and enhanced healing of damaged mucosa. The microbe must be of human origin for host-specific interactions by the probiotic and it must of course be safe for the host. It must also have good technological properties such as strain stability and oxygen tolerance, and must be able to be produced on an industrial scale. Last but not least, the intended probiotic strain must have a documented health effect.

### 1.4.1 Health effects of probiotics

The use of probiotics has been linked to a number of positive health effects. These effects can be divided into well-documented outcomes with good evidence according to meta-analyses and promising, but not fully proven, health effects with low evidence or where no meta-analyses have been performed. Well-documented health effects with good evidence include:

- Lower frequency and duration of diarrhoea connected to use of antibiotics (Johnston *et al.*, 2011) or caused by an infectious agent (Allen *et al.*, 2010)
- Reduction of *Helicobacter pylori* infection (Tong *et al.*, 2007)
- Relief from constipation (Dimidi *et al.*, 2014)
- Relief from irritable bowel syndrome (IBS) (Ortiz-Lucas *et al.*, 2013)
- Reduction of total and LDL cholesterol plasma concentrations (Guo *et al.*, 2011)
- Treatment of colic symptoms in breastfed infants (Anabrees *et al.*, 2013).

Health effects for which further evidence is needed include:

- Prevention of allergic disease and food hypersensitivity (Osborn & Sinn, 2007)
- Maintenance of remission in ulcerative colitis (Naidoo *et al.*, 2011; Sang *et al.*, 2010) and Crohn's disease (Butterworth *et al.*, 2008)
- Treatment of colic symptoms in formula-fed infants (Anabrees *et al.*, 2013).

In 2002, more than 19 species from seven different genera of bacteria and yeast were used as probiotics and had documented health effects (Ouwehand *et al.*, 2002). However, the list of microbes used in probiotic products has grown continually since then and the exact number is currently unknown. The most commonly used strains belong to the LAB, but there are also species from the genera *Escherichia*, *Bacillus* and *Propionibacterium*. What is important to know is that the health effects seen when using microbes in probiotic products are usually strain-specific and that there can be a huge difference in probiotic potential even within a single species of microbes.

### 1.4.2 Lactic acid bacteria (LAB)

The group of LAB consists of Gram-positive cocci or rod-shaped bacteria, which are non-sporulating and non-respiring. The name 'lactic acid bacteria' is

used since they all produce lactic acid as the major end-product during fermentation of carbohydrates. Genera included in this group are: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. The first pure culture of an LAB was obtained in 1873, but the systematic classification of the group remained slightly unclear until 1919 and the work of Orla-Jensen. The characteristics used by Orla-Jensen as the basis for classification included morphology (cocci or rods), mode of glucose fermentation (homo- or hetero- fermentation), minimum, maximum and optimum growth temperature and range of sugar utilisation. These characteristics are still used in the classification of LAB with a few additions, *e.g.* configuration of the lactic acid produced, ability to grow at high salt concentrations, acid or alkaline tolerance and fatty acid composition and constituents of the cell wall (Axelsson, 2004).

The industrial use of LAB has changed greatly through the years, but they have always been important as starter cultures in dairy processes. These starters can be single-strain, multiple strains of one species, multiple strains of different species or raw mixed strains where the strains are partly known or unknown. Starter cultures contribute to the final product by changing the sensory experience and also extend the shelf life of the food item. This is done by flavour synthesis, pH reduction, protein hydrolysis that causes changes in texture and taste, synthesis of texturising agents which influence the consistency of the product and production of inhibitory components. These characteristics and activities are also important in the fermentation of meat, vegetable and cereal products (Mäyrä-Mäkinen *et al.*, 1993). In bread making, the fermentation by LAB has a number of different functions, including making the dough easier to bake, giving a more appealing and softer bread crumb and control and inhibition of contaminating or spoiling microorganisms during the fermentation and in the bread *e.g.* elongation of mould-free time during storage of bread (Salovaara *et al.*, 1993). LAB can also contribute to a better end-product by producing flavour, aromas and increasing mineral availability, as mentioned earlier. Different species of LAB belonging to at least three different genera (*Bifidobacterium*, *Enterococcus* and *Lactobacillus*) are commonly used as probiotic supplements and the majority of these belong to the genus *Lactobacillus* (Ouwehand *et al.*, 2002).

### *1.4.3* The genus *Lactobacillus*

The genus *Lactobacillus* belongs to the phylum Firmicutes, the class Bacilli, the order Lactobacillales and the family Lactobacillaceae. Lactobacilli are rod-shaped, oxygen-resistant anaerobes which are chemo-organotrophic and

require a rich medium to grow. They can be found in a variety of different environments ranging from foods to the respiratory, GI and genital tract of humans and animals, sewage and plant material. This genus is among the largest of the bacterial genera, with approximately 225 known species (Euzéby, 2014-11-07), one of which is the GI-tract associated and probiotic species *Lactobacillus reuteri*.

### *1.4.4 Lactobacillus reuteri*

*Lactobacillus reuteri* was first described as a species by Kandler *et al*. (1980). However, back in 1962 it had been recognised as a distinct "type" and was then regarded as *Lactobacillus fermentum* biotype II (Kandler *et al.*, 1980). *Lactobacillus reuteri* is a rod-shaped bacterium which normally resides in the GI tract of humans and a number of other animal species, including poultry, pigs, dogs, rodents *etc*. (Hammes & Hertel, 2006). In the gut of pigs, rodents and chickens, *L. reuteri* seems to be one of the most abundant species and has been shown to be present in a large subset of animals (Walter *et al.*, 2011). The prevalence of *L. reuteri* in the human gut seems to be much lower, however, and it is only detected occasionally. Despite this, it is present not only in the GI tract of humans, but also in breast milk, the oral cavity and the vagina. In addition to being a stable member of the microbiota in humans and animals, *L. reuteri* has also frequently been isolated in high temperature sourdoughs. However, these isolates are believed to originate from faecal contaminants of rodent origin (Su *et al.*, 2012). Through being present in so many different warm-blooded animals, *L. reuteri* has adapted to being highly host-specific in terms of phenotypic characteristics. Its different phenotypic characteristics include *e.g*. ability to adhere to epithelial cells and production of certain enzymes or anti-microbial substances (Walter *et al.*, 2011). Like other lactobacilli, *L. reuteri* is a highly demanding species and requires easily fermentable sugars, amino acids, vitamins and nucleotides. With all these requirements met, *L. reuteri* has a duplication time of less than one hour (Gerez *et al.*, 2008). The species uses the phosphoketolase pathway for fermentation of carbohydrates to form lactate, acetic acid, ethanol and carbon dioxide. This pathway has low energetic yield, but *L. reuteri* uses external electron acceptors such as fructose, glycerol, pyruvate, citrate, nitrate and oxygen to improve this. Even though it is a highly demanding species, *L. reuteri* has been shown to resist and survive in environments with low pH and in the presence of bile salts, which are important characteristics for a probiotic strain (Whitehead *et al.*, 2008; Jacobsen *et al.*, 1999).

Other characteristics of *L. reuteri* that make it a good candidate as a probiotic strain include the fact that it can compete with other microorganisms

by the production of several antimicrobial substances. Lactic acid and acetic acid, which are produced during normal carbohydrate degradation, can inhibit the growth of microbes and are especially potent against Gram-negative bacteria. In addition to these normally produced metabolites, some strains of *L. reuteri* are known to produce a broad-spectrum antimicrobial metabolite named reuterin. This compound is a low molecular weight, water-soluble, non-proteinaceous and neutral end-product associated with the fermentation of glycerol to 1,3-propandiol and can be accumulated and secreted by *L. reuteri* under certain conditions (Spinler *et al.*, 2008). Reuterin is a mixture of 3-hydroxypropionaldehydes (3-HPA) and includes a monomer, a hydrated monomer and a cyclic dimer. It is formed during anaerobic growth by a two-step pathway where glycerol is first dehydrated to form reuterin, some of which is further reduced to 1,3-propanediol. The reduction of reuterin to 1,3-propanediol is proposed to generate $NAD^+$ from NADH and hence contribute to improved growth (Schaefer *et al.*, 2010). It has been shown that the presence of other bacteria such as *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Staphylococcus*, *Clostridium* and *Pseudomonas* stimulates this conversion of glycerol to reuterin and that it provides *L. reuteri* with a competitive advantage in the intestinal tract (Schaefer *et al.*, 2010; Chung *et al.*, 1989). Reuterin has been proven to inhibit the growth of Gram-negative and Gram-positive bacteria, as well as yeasts, fungi and protozoa, and also to have an effect against viruses (Walter *et al.*, 2011; Schaefer *et al.*, 2010; Jacobsen *et al.*, 1999; Axelsson *et al.*, 1989). In addition, some strains of *L. reuteri* have been observed to produce yet another low molecular weight antimicrobial substance named reutericyclin, a tetramic acid that has been shown to be bacteriostatic or bactericidal to Gram-positive bacteria. However, Gram-negative bacteria and yeast seem to be resistant to reutericyclin due to the barrier properties of their outer membrane (Gänzle *et al.*, 2000).

Numerous clinical studies have proven the potential of *L. reuteri* as a probiotic species. Studies have shown that supplementation with *L. reuteri* can alleviate GI tract symptoms and improve colicky symptoms in breastfed infants (Indrio *et al.*, 2014; Savino *et al.*, 2010), reduce infections (Tubelius *et al.*, 2005; Weizman *et al.*, 2005) and improve feeding tolerance in formula-fed premature neonates (Indrio *et al.*, 2008). Other studies have reported promising results in treating children with ulcerative colitis (Oliva *et al.*, 2012) and decreasing the number of days with diarrhoea in children treated with a daily supplement compared with untreated groups (Gutierrez-Castrellon *et al.*, 2014; Weizman *et al.*, 2005). Children with acute rotavirus-induced diarrhoea also have a reduced duration of watery diarrhoea when supplemented with *L. reuteri* (Shornikova *et al.*, 1997). In addition to having an effect on infants and

children, *L. reuteri* has been shown to suppress infections caused by *Helicobacter pylori* in adults. Treatment with *L. reuteri* reduced the overall occurrence of dyspeptic symptoms, abnormal defecation and flatulence in these patients compared with a group receiving a placebo (Francavilla *et al.*, 2014; Francavilla *et al.*, 2008). Besides being able to reduce the occurrence of symptoms in various diseases and disorders, *L. reuteri* has been proven to reduce the transport of live bacteria from the intestinal lumen to the bloodstream and internal organs of the body (Wang *et al.*, 1995). This phenomenon, called bacterial translocation, can be caused by bacterial pathogens or by members of the normal gut microbiota when the host is exposed to stressful conditions such as starvation, trauma *etc*. Studies have also shown a positive effect in maintaining gut mucosal integrity after treatment with *L. reuteri* (Adawi *et al.*, 1997).

## 1.5 Synbiotics and production of bioactive compounds

The term 'synbiotic' is used when a product contains both probiotics and prebiotics and was coined at the same time as the term prebiotics in 1995 (Gibson & Roberfroid, 1995). The definition of a synbiotic is still under revision, since there is some divided opinion regarding whether the prebiotic included in the synbiotic formulation should promote the growth and/or activity of the partner probiotic. There have been a number of different studies on synbiotic formulations to investigate whether the viability and persistence of a probiotic bacterium could be enhanced by supplementing it with a prebiotic compound. Studies on mice have shown that different kinds of prebiotics, *e.g*. soybean oligosaccharide, fructooligosaccharides and inulin, can significantly enhance the survival and number of bacteria, as well as prolonging the retention time of probiotic strains such as *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus casei* (Su *et al.*, 2007). On the other hand, clinical studies on humans have shown that a synbiotic formulation can lead to an increase in a completely different species than the probiotic supplied in the synbiotic formulation itself (Piirainen *et al.*, 2008). These studies show the importance of having a well-established synbiotic formulation, but also that the effect can be difficult to predict because of the complex environment in the intestinal tract. As mentioned in the previous section, probiotics have been shown to improve some of the symptoms related to IBS. However, there have been very few studies published indicating that the use of prebiotics alone could have the same effect. Combinations of probiotics and prebiotics have been shown to decrease a number of different symptoms in IBS patients, including nausea, flatulence and

colitis (Bittner *et al.*, 2005). Other studies have shown that combinations of lactobacilli, bifidobacteria and fructooligosaccharides can reduce the incidence of common infectious diseases in children during the winter period and thereby the number of days the children have to stay away from school (Cazzola *et al.*, 2010). Effects have also been seen on the plasma cholesterol levels in human subjects, where a synbiotic formulation containing lactobacilli and inulin reduced the total and LDL cholesterol levels (Ooi *et al.*, 2010).

In addition to enhancing the growth and persistence of bacteria, it might also be possible to enhance the effect of probiotics on human health by enhancing the production of certain health promoting agents or bioactive compounds by the probiotic bacteria. Bioactive compounds (also referred to as nutraceuticals) are naturally occurring substances that are part of the diet and can be essential or non-essential, but should have an established effect on human health. They can be isolated nutrients or parts of dietary supplements, genetically engineered 'designer' foods, herbal products or processed foods, *e.g*. cereals, soups and beverages (Biesalski *et al.*, 2009). Bioactive compounds can be present in different food ingredients, but they are not always available to the host. In cereals, the phenolic acids present are mainly bound to arabinoxylans through ester bonds and these fibre-bound phenolic acids cannot be hydrolysed by human digestive enzymes (Saura-Calixto, 2010). Studies have shown that probiotic bacteria such as *L. reuteri* can release these phenolic acids and possibly make them accessible to the host. Barley and oat flour fermented by different species of lactobacilli, including *L. reuteri*, can increase the bioavailability of certain dietary phenolic acids such as caffeic, coumaric, ferulic and sinapic acid (Hole *et al.*, 2012). Similar results have been seen for cowpea flour fermented with *L. plantarum*, which leads to an improvement in phenolic compound concentration and antioxidant activity (Dueñas *et al.*, 2005), and in rye flour fermented with baker's yeast, *L. brevis* and *L. plantarum*, which gives higher concentrations of folate and phenolic compounds (Liukkonen *et al.*, 2003). Concentrations of other bioactive compounds such as γ-aminobutyric acid (GABA) have also been shown to increase on fermenting different kinds of cereals or sub-cereals with lactobacilli (Stromeck *et al.*, 2011; Coda *et al.*, 2010). Another effect is the production of SCFA, which are associated with reduced risk of diseases such as cancer, cardiovascular disease and inflammatory bowel diseases (Floch & Hong-Curtiss, 2002; Jenkins *et al.*, 1999).

## 1.6 For a healthy intestine

In sheer cell numbers, the human body consists to a large degree of its microbiome, which is "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg & Mccray, 2001). The human body consist of around ten times as many bacterial as human cells (Savage, 1977). In the gastrointestinal tract alone more than 400 bacterial species have been found, but more recent studies claim that there could be as many as 1000 different species of bacteria present (Rajilić-Stojanović *et al.*, 2007). Most of these bacteria are found in the GI tract, ranging from $10^3$ colony forming units (CFU) per ml gastric juice in the stomach and increasing throughout the intestine, reaching the highest concentrations ($10^{12}$ CFU per gram faeces) in the colon (Mitsuoka, 1992). One reason that the GI tract can house such a great number of bacterial cells is because it is one of the body's largest external surfaces, measuring a total of 32 $m^2$, the size of half a badminton court (Helander & Fändriks, 2014). In human foetuses these surfaces have long been considered to be sterile until birth and it was once widely accepted that the colonisation of the human body occurs during and after birth (Mackie *et al.*, 1999). However, more recent studies suggest that there is some exchange between the mother and foetus, suggesting that the foetus might not be completely sterile (Jiménez *et al.*, 2008). The composition of the intestinal microbiota is set at an early age (Palmer *et al.*, 2007) and is considered to remain relatively stable in a healthy person throughout life (Rajilić-Stojanović *et al.*, 2013). However, the composition can be disturbed and changed by different factors, such as physiological state, drugs, disease, diet and stress (Lozupone *et al.*, 2012; Mitsuoka, 1992). The importance of having a stable intestinal microbiota to avoid disease has been shown in studies as far back as the 1950s on mice and guinea pigs. Bohnhoff *et al.* (1954) showed that oral administration of antibiotics to mice made them more susceptible to *Salmonella* infections (Schrezenmeir & de Vrese, 2001). Similar experiments performed in 1955 on guinea pigs showed that administration of a specific antibiotic rendered the animals more susceptible to *Vibrio cholerea* (Freter, 1955).

In addition to playing an important role against pathogenic microorganisms, changes or differences in the composition of the microbiome have been linked to a number of different diseases and syndromes. Obesity, circulatory disease, inflammatory bowel disease and autism are just a few examples of diseases or physical or psychological states where a difference in the microbiome composition has been seen compared with healthy subjects (Finegold, 2008; Holmes *et al.*, 2008; Marchesi *et al.*, 2007; Turnbaugh *et al.*, 2006). The intestinal microbiome has also been shown to have an influence on drug

metabolism and toxicity, dietary calorific bioavailability and recovery after surgery (Kinross *et al.*, 2010; Clayton *et al.*, 2006; Hooper & Gordon, 2001). One effect that disruption of the normal microbiome or dysbiosis can lead to is a syndrome called 'leaky gut', which in turn has been linked to a number of different diseases (Frazier *et al.*, 2011; Sartor, 2008).

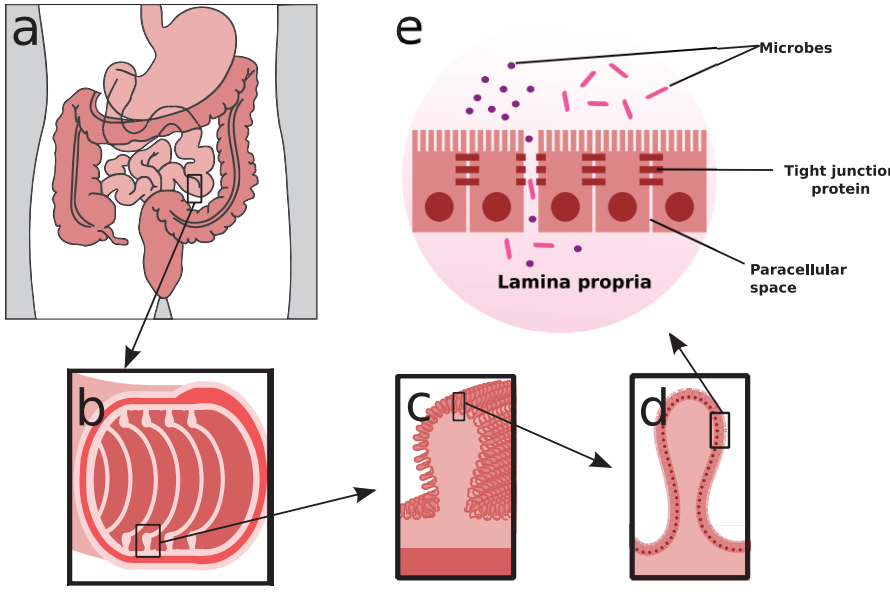


*Figure 3.* Simplified overview of the structure of a small intestine, showing a) the gastrointestinal tract, b) intersection of the small intestine with its folds, c,d) villi and e) individual epithelial cells with microvilli. The epithelial layer is comprised of a single cell layer where the paracellular space is sealed by tight junction proteins (TJ) the function of which is to limit the leakage of unwanted substances or microorganisms. A leaky gut, or increased intestinal permeability, can be caused by TJ with a disturbed function, leading to microbes entering the lamina propria.

### 1.6.1 Leaky gut

The more scientific term for leaky gut is increased intestinal permeability and it refers to the intestine's ability to prevent harmful substances from entering the internal environment of the body or, in the case of disease, the inability of the intestine to prevent leakage of these harmful substances (Bjarnason *et al.*, 1995). The gastrointestinal wall consists of different barriers to prevent unwanted substances or microorganisms from entering the blood stream. The epithelial layer itself consists of two barriers (see Figure 3), the first of which is the cell membrane itself. The cell membrane consists of lipids and does not allow water-soluble substances to pass through. The second barrier can be found between each cell, in an area called the paracellular space which is controlled by proteins called tight junction (TJ) proteins. These can open and

close to allow fluids, nutrients and even microorganisms to cross from the lumen into the lamina propria of the intestine (Madara & Pappenheimer, 1987). Tight junctions with a disturbed function caused *e.g.* by a disease result in increased intestinal permeability or leaky gut.

In the 1980s, Crohn's disease and coeliac disease were investigated in order to establish whether they caused increased intestinal permeability. These early studies revealed that patients suffering from Crohn's disease had increased intestinal permeability to larger-sized molecules such as lactulose. However, no change in the permeability of smaller molecules, such as mannitol or rhamnose, was seen in these patients (Ukabam *et al*., 1983; Pearson *et al*., 1982). A similar increase in permeability to larger-sized molecules was seen in patients with coeliac disease, while for smaller molecules such as mannitol a decrease in permeability was seen in these patients (Ukabam & Cooper, 1984; Bjarnason *et al*., 1983). By eliminating gluten from their diet, the intestinal permeability of these patients was normalised. Other disorders and diseases have also been suggested to be connected to an increase in intestinal permeability, including food allergies, asthma and some allergic skin conditions. In addition, patients with severe general trauma, patients who have undergone major surgery and burn patients have been demonstrated to have abnormalities in intestinal permeability (Hollander, 1999). Moreover, an increase in intestinal permeability has been reported in patients suffering from other gastrointestinal disorders. For example, patients suffering from diarrhoea-predominant IBS have been shown to have a higher ratio of lactulose/mannitol in their urine samples compared with controls, suggesting greater leakage of lactulose. Several studies have reported this increase in permeability in patients with IBS and some have even reported correlations between an increase in permeability and abdominal pain (Zhou *et al*., 2009; Dunlop *et al*., 2006; Spiller *et al*., 2000). Understanding and being able to measure intestinal permeability is important in prevention and early detection of all these diseases and disorders. Protection against a reduction in the protective barrier function is highly important, since it is involved in *e.g.* several gastrointestinal diseases and allergies.

The possible role of probiotics in decreasing intestinal permeability in patients suffering from different diseases and disorders has been evaluated in a number of studies. Supplements of probiotic lactobacilli (*L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246) given to children with atopic dermatitis resulted in a decrease in gastrointestinal symptoms and intestinal permeability in patients with severe to moderate atopic dermatitis compared with the placebo group (Rosenfeldt *et al.*, 2004). Treatment of children suffering from mild to moderately active Crohn's disease with *Lactobacillus rhamnosus* GG resulted

in a lower disease score and an improvement in intestinal barrier function, with lower permeability (Gupta *et al.*, 2000). Pre-term infants, who have been shown to have increased intestinal permeability, exhibited a decrease in permeability after treatment with *B. animalis* subsp. *lactis*-supplemented pre-term formula (Prenan Nestlé BLSPF) (Stratiki *et al.*, 2007). Probiotics including *L. plantarum* and *L. reuteri* have also been shown to prevent the increased intestinal permeability caused by *Escherichia coli* in mice and in intestinal epithelial cell models (Liu, 2013; Mangell *et al.*, 2002).

# 2 Aims

The use of probiotics is increasing among the public. Although these are still classified as foods or food supplement, they have been shown to be effective in the prevention and treatment of a number of diseases and conditions as described in the previous chapter. The main hypothesis examined in this thesis was that in order to exploit the full potential of a probiotic bacterium, it needs to be metabolically active in the intestine and that the probiotic effect can be enhanced if appropriate nutritional compounds are provided to *L. reuteri*. In order to test this main hypothesis, three sub-hypotheses were formulated: 1) *L. reuteri* grows well in cereal substrates and produces bioactive compounds; 2) production of these bioactive compounds is strain-specific and can be enhanced by using certain cereal varieties; and 3) some of these bioactive compounds have a protective effect against increased intestinal permeability. To test these hypotheses, the following objectives were established:

- Isolation of new *L. reuteri* strains from sourdough fermented at high temperature (**Paper I**)
- Typing and characterisation of isolated strains (**Paper I**)
- Comparison of the growth of *L. reuteri* strains of different origins on different cereal varieties (**Paper I**)
- Comparison of metabolites of each strain in cereal varieties to find possible bioactive compounds (**Paper I**)
- Determination of the effect of cereal-reuteri combinations on intestinal permeability using a cell culture model (**Paper II**)
- Correlation of possible effects in the cell culture model to the metabolic profile of the cereal-reuteri combinations (**Paper II**).

# 3 Results and discussion

Previous studies have established that *L. reuteri* can be isolated from industrial sourdoughs and that they can even dominate these sourdoughs (Meroth *et al.*, 2003). Studies have also been conducted on the growth and metabolism of *L. reuteri* in different kinds of cereal, many of these studying the production of bioactive compounds during the fermentation of cereals. Since the occurrence of *L. reuteri* is common even when using spontaneously fermented dough, one of the first objectives in this thesis was to isolate and if possible identify the origin of these strains.

## 3.1 Isolation of *L. reuteri* strains from cereals (Paper I)

A total of 15 different flours were analysed for the presence of lactobacilli and more specifically *L. reuteri*. Flours made from different cereals (barley, buckwheat, millet, rye, sorghum and wheat) were used, as well as both conventionally grown and organic flours. Most of the flours (nine) were Swedish brands, four were German and two were Chinese. Flour-water mixtures were prepared and incubated at 37 and 40 ºC. Isolation and identification were performed using selective medium (Rogosa-vancomycin), reuterin-specific PCR and finally 16S rRNA gene sequencing. *Lactobacillus reuteri* could not be detected or isolated from any of the nine Swedish flours or three of the German flours. However, in the two Chinese flours (buckwheat and millet) and in one German flour (organic rye), *L. reuteri* was detected by PCR. After isolation on selective medium, a total of 18 colonies were collected and identified using reuteri-specific PCR. These isolates were divided into five groups using genomic fingerprinting (rep-PCR) and later identified as *L. reuteri* by 16S rRNA gene sequencing (Table 2 in Paper I). According to these results, the occurrence of *L. reuteri* in flour seems to depend on the geographical origin of the flour (Paper I). Previous studies have reported the

presence of *L. reuteri* in only a few different cereals (rye, teff and sorghum) from a few countries (Denmark, Germany, Ireland and Sudan) (De Vuyst *et al.*, 2014).

## 3.2 Characterisation and phylogenetic positioning of isolated *L. reuteri* strains (Paper I)

In order to determine the possible origin of the strains isolated from fermented cereals, a number of different analyses were performed. Since some strains of *L. reuteri* can produce the antimicrobial substance reuterin and this ability to some extent correlates with the origin of the strain, a reuterin assay was performed on all isolates. A PCR for analysing the presence of a gene (*pduC*) involved in the production of reuterin and a gene (*ureC*) involved in the production of urease was performed (since these genes are also correlated with the origin). To further characterise and compare the new strains with strains of other origin, multi-locus sequence analysis (MLSA) was performed on one isolate from each of the five groups and compared against a total of 84 previously isolated strains of different animal origin. In this analysis, the sequences of seven house-keeping genes were compared, as previously performed on *L. reuteri* strains isolated from different animals and also fermented cereals.

All isolates gave a negative result in the reuterin assay and for the presence of the gene *pduC*, but a positive result for the presence of the gene *ureC* (Table 2 in Paper I). Other studies have shown that strains of rodent origin generally test negative for reuterin and positive for urease (Walter *et al.*, 2011). The MLSA showed that all strains clustered together with strains of rodent origin, two (strain SU7-3 and SU12-3) within clade I and three (strain SU3-3, SU17-3 and SU18-3) within clade III (Figure 1 in Paper I). Since the MLSA, urease PCR and reuterin PCR results all showed that the new strains were similar to strains of rodent origin, it is likely that faecal contamination by rodents occurred during storage of the cereals. *Lactobacillus reuteri* is mainly found in the intestine of different animals and has never been isolated from plants or plant-derived products except sourdoughs, and therefore this conclusion is plausible. The possible rodent origin of strains isolated from sourdough has been noted in other studies using MLSA, which also showed that *L. reuteri* contamination could be of human origin (Su *et al.*, 2012).

## 3.3 Growth and pH reduction of *L. reuteri* in barley flour (Paper I)

It is already a well-established fact that *L. reuteri* grows well in different cereals, including barley, oat, rye and wheat (De Vuyst *et al.*, 2014; Hole *et al.*, 2012; Charalampopoulos *et al.*, 2002). Only a few studies have compared the growth of single strains of *L. reuteri* to that of other species of lactobacilli in different cereals. One of these studies has shown that *L. reuteri* (NCIMB 11951) grows to a lower maximum density than *L. plantarum* (NCIMB 8826), *L. fermentum* (NCIMB 12116) and *L. acidophilus* (NCIMB 8821) in barley- and wheat-based substrates (Charalampopoulos *et al.*, 2002). On the other hand, another study has shown that *L. reuteri* (LTH2584) can grow to similar densities as *L. sanfranciscensis* (LTH2581) in wheat-based substrates (Gänzle & Vogel, 2003). Differences (as much as 2 $\log_{10}$) in final growth density of *L. reuteri* have been reported in different studies using similar cereal substrates. Since no single study has analysed the growth of more than one strain of *L. reuteri* in cereal substrates, questions regarding the growth of *L. reuteri* in cereals remain unanswered. To answer questions regarding strain specificity in growth and activity in cereal substrates, in this thesis strains of different origin grown in slightly different cereal substrates were compared. In the analysis (Paper I), six barley varieties with slightly different carbohydrate composition were available as milled flour. Differences were mainly seen in fibre and starch content and also in starch, with different amylose/amylopectin ratio (Table 1 in Paper I). In addition to the six barley varieties, a total of six bacterial strains were selected for analysis. These comprised: Three *L. reuteri* strains isolated from sourdoughs, a strain representing each rodent clade (SU12-3 and SU18-3) and a strain previously isolated from sourdough (LTH 5531, a gift from Christian Hertel); two *L. reuteri* strains (ATCC PTA 6475 and DSM 17938) which are commercially available probiotic strains of human origin; and, in order to allow comparison with another species and determine whether growth and activity was species-specific, one strain of *L. plantarum* (36E).

In order to analyse the activity of the added bacteria specifically and not the combined effect of the added bacteria and the endogenous microbiota and enzymes, the flour was also heat-treated to inactivate the enzymes and microorganisms. In all, a total of 72 combinations of barley and bacteria were analysed. Mixtures of water and flour were inoculated with overnight cultures of each bacterium, incubated at 37 ºC and subjected to back-slopping three times before the actual analysis. A sample was taken at the start and after 3, 6 and 24 hours to analyse bacterial growth and pH. After the last sampling, the sourdoughs were centrifuged and the supernatant was collected for use in chemical analysis (Figure 4).

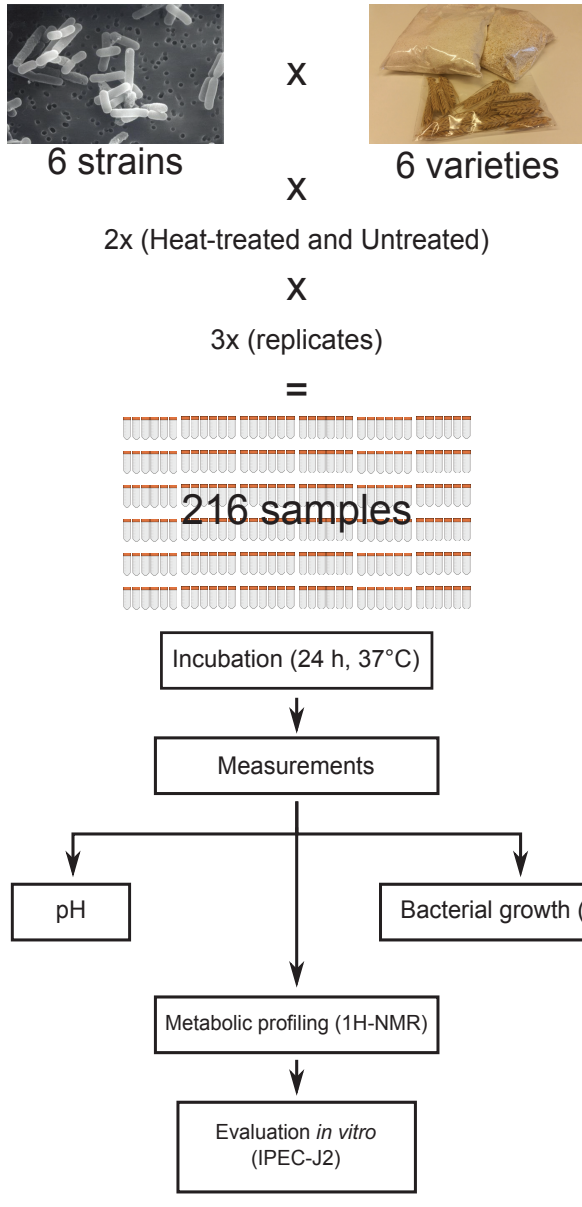


*Figure 4.* Schematic overview of the experimental procedure used in this study. Whole grain flours from six barley varieties were heat-treated and left untreated before fermentation by six bacterial strains. Fermentation ran for 24 hours at 37 °C and was performed in triplicate, giving a total number of 216 samples. Samples were subjected to different measurements including pH, bacterial growth followed by metabolic profiling and evaluation in an *in vitro* model using small intestinal epithelial cells (IPEC-J2).

### 3.3.1 Bacterial counts

All strains grew well in both untreated and heat-treated flour, reaching a final bacterial cell count of 9.0-9.7 $\log_{10}$ CFU/g in untreated flour and 7.3-9.3 in heat-treated flour (Table 3 in Paper I). Statistical analyses were performed to identify significant differences in growth between bacteria in different barley varieties.

All strains grew better in untreated than in heat-treated flour, but *L. reuteri* seemed to grow less well in heat-treated flour than *L. plantarum* 36E. Since some enzymes, *e.g*. amylase, were inactivated during the heat treatment, lower amounts of monosaccharides, *e.g*. glucose, maltose and fructose, should have been made available to the bacteria. Most of the carbohydrates in cereals are in the form of polysaccharides and oligosaccharides such as starch, beta-glucan and fructan, all of which, as far as we know, *L. reuteri* cannot degrade. These two factors could explain the lower growth seen in heat-treated flour. The analysis also revealed similarities and differences at strain level. In untreated flour the differences in growth were less pronounced than in heat-treated flour, with growth density varying by 0.5 $\log_{10}$ CFU/g at most between untreated flour from different barley varieties. In heat-treated barley flour larger variations in growth density could be seen. This was especially the case for DSM 17938, for which growth density ranged from 7.3-8.8 $\log_{10}$ CFU/g, a difference of 1.5 $\log_{10}$, while the other strains showed differences in growth of 0.4-0.7 $\log_{10}$. This could suggest that DSM 17938 is more dependent on available monosaccharides and disaccharides than the other *L. reuteri* strains and hence relies more on the presence and activity of enzymes in the flour for the degradation of polysaccharides and oligosaccharides.

### 3.3.2 pH

The reduction in pH was also significantly different when the bacteria were grown in heat-treated compared with untreated flour. In untreated flour, the pH reached 3.4-3.7 and in heat-treated flour it reached 3.9-5.3 after 24 hours of fermentation. As found for bacterial counts, the pH was lower in heat-treated flours fermented with *L. plantarum* 36E than with *L. reuteri* strains. Growth to a lower density is correlated with higher pH for lactobacilli, since if the bacteria have lower amounts of available monosaccharides they produce lower amounts of lactate and acetate as end-products. Significant differences in pH were found between strains of *L. reuteri* in both untreated and heat-treated barley flour (Figure 2 in Paper I). Comparing the pH reduction in each strain alone in the barley varieties did not reveal any differences in untreated flour (Table 3 in Paper I). However, differences were found in heat-treated flour when comparing the pH in all the barley varieties for each strain (Table 4 in

Paper I), suggesting that the difference in pH reduction is greater between barley varieties when heat-treated flours are fermented. These results suggest that the production of acids is both species- and strain-specific, at least when the bacteria are grown on heat-treated barley flour. The differences in pH seen in heat-treated flours could be due to differences in available sugars. The endogenous enzymatic activity in untreated barley flour causes a steady flow of monosaccharides to be made available from the degradation of *e.g.* starch. The inactivation of enzymes in heat-treated flour leaves the bacteria with only the free monosaccharides and disaccharides, however, since *e.g.* no amylases are available to degrade the starch. Enzymes with the ability to hydrolyse starch, one of which is called pullulanase, are present in some species of *Lactobacillus*. Pullulanase is able to produce maltose, amylose and glucose by debranching starch. In *L. reuteri*, the only form of this type of enzymes that has been detected so far is neopullulanase and the presence of the gene for this enzyme seems to be strain-specific, *e.g.* it is present in ATCC PTA 6475, but not in ATCC 55730 (Saulnier *et al.*, 2011). Neopullulanase has been reported to be able to hydrolyse pullulan, but whether it has a function in the degradation of starch and what that function is remain to be determined. Some studies claim that it is important in the degradation of starch, while others claim that it does not have any effect on starch (Hii *et al.*, 2012; Saulnier *et al.*, 2011; Park *et al.*, 2000). The presence of the gene for neopullulanase in the other strains used in this study and expression of this enzyme could help explain some of the differences in growth in heat-treated flour observed between strains.

## 3.4 Chemical analysis of barley fermented with *L. reuteri* (Paper I)

After 24 hours of incubation, the fermented barley flours were centrifuged and the supernatant was collected for use in chemical analysis. The choice of using 1H-NMR as the method for analysing the different samples was based on the fact that it would give the best overview of metabolites produced during fermentation. A total of 216 pH-adjusted samples were analysed and metabolites were manually identified using the NMR spectra of known compounds in the compound library of the software Chenomx NMR suite, as well as using data available in internet browser databases such as the Human Metabolome Database (HMDB) and the Biological Resonance Data Bank. Results consisting of name and concentrations of identified compounds were analysed using multivariate statistics to evaluate differences between samples and find possible correlations (Figures 3-5 in Paper I). Compounds responsible

for the separation of groups of samples were further analysed by ANOVA to identify significant differences between individual samples.

Table 1. *Final growth density of L. reuteri strains in untreated and heat-treated flour in relation to sugar content (SC), electron acceptor content (EAC) and total amino acid content (AAC) in unfermented barley flour. Growth data are mean values of samples taken after 24 hours incubation and are given as $log_{10}$ CFU/g. Values sharing footnote letters are not significantly different (Student's t-test at alpha 0.05). SC = sum of the concentrations (mM, in 5-fold diluted sourdough solution) of glucose, maltose and sucrose. EAC = sum of the concentrations of fructose, fumarate and malate. AAC = sum of concentrations of alanine, asparagine, aspartate, isoleucine, leucine, phenylalanine, proline, tyrosine and valine.*

| | Barley variety | DSM 17938 | ATCC PTA 6475 | LTH 5531 | SU12-3 | SU18-3 | SC | EAC | AAC |
|---|---|---|---|---|---|---|---|---|---|
| Untreated | 120 | $9.2^{BC}$ | $9.6^{A}$ | $9.5^{AB}$ | $9.5^{AB}$ | $9.0^{C}$ | 15.8 | 5.5 | 2.4 |
| | 155 | $9.5^{A}$ | $9.6^{AB}$ | $9.5^{A}$ | $9.4^{BC}$ | $9.1^{BC}$ | 21.9 | 7.4 | 2.5 |
| | 181 | $9.5^{A}$ | $9.7^{A}$ | $9.7^{A}$ | $9.6^{A}$ | $9.5^{A}$ | 25.0 | 6.1 | 4.1 |
| | 224 | $9.4^{AB}$ | $9.6^{A}$ | $9.5^{A}$ | $9.6^{A}$ | $9.3^{AB}$ | 13.8 | 6.0 | 2.3 |
| | 228 | $9.5^{A}$ | $9.6^{AB}$ | $9.5^{A}$ | $9.4^{BC}$ | $9.4^{A}$ | 20.5 | 5.3 | 1.6 |
| | 249 | $9.0^{C}$ | $9.4^{B}$ | $9.2^{B}$ | $9.2^{C}$ | $9.1^{BC}$ | 15.7 | 2.3 | 1.4 |
| Heat-treated | 120 | $7.3^{D}$ | $8.4^{C}$ | $8.1^{C}$ | $8.3^{BC}$ | $8.1^{B}$ | 2.2 | 1.6 | 0.2 |
| | 155 | $8.4^{B}$ | $8.7^{AB}$ | $8.7^{A}$ | $8.6^{ABC}$ | $8.4^{AB}$ | 3.1 | 0.7 | 0.4 |
| | 181 | $7.9^{C}$ | $8.8^{A}$ | $8.8^{A}$ | $8.7^{AB}$ | $8.6^{A}$ | 4.7 | 1.7 | 1.6 |
| | 224 | $8.8^{A}$ | $8.5^{BC}$ | $8.7^{A}$ | $8.7^{A}$ | $8.6^{A}$ | 5.4 | 1.1 | 0.7 |
| | 228 | $7.8^{C}$ | $8.9^{A}$ | $8.6^{AB}$ | $8.4^{ABC}$ | $8.4^{AB}$ | 3.2 | 2.1 | 0.4 |
| | 249 | $7.3^{D}$ | $8.6^{BC}$ | $8.3^{BC}$ | $8.2^{C}$ | $8.3^{AB}$ | 1.6 | 1.3 | 0.2 |

### 3.4.1 Metabolic profiling by 1H-NMR

Using the compound library and data available in the internet browser databases, a total of 29 different compounds were identified in the fermented barley samples (Table 5 in Paper I). Identified compounds included major metabolites such as acetate, ethanol, lactate, mannitol and succinate, as well as monosaccharides and disaccharides such as fructose, glucose, maltose and sucrose. These compounds were expected, since they are part of the metabolic pathway for fermenting simple carbohydrates used by *L. reuteri* (Figure 5). Comparing the results from unfermented barley flours revealed which compounds were produced and which were consumed. In untreated flour the main source of energy seemed to be glucose, followed by maltose, since the consumption of these two monosaccharides was highest. As in previous studies on *L. reuteri* from sourdoughs, the strains used in Paper I seemed to use

fructose, fumarate and malate as electron acceptors, forming mannitol and succinate (Stolz *et al.*, 1995). Even though samples from fermented barley still contained fructose and in some cases fumarate and malate, the strains produced ethanol in similar concentrations to lactate. The production of ethanol by LAB leads to a lower net gain of ATP per glucose consumed (Axelsson, 2004), but electron acceptors are produced instead of ATP and can later be used to achieve higher energy yields. There are advantages that might explain the increase in ethanol production seen in barley flour fermented by *L. reuteri*. Higher concentrations of ethanol in the barley flour could give an competitive advantage over other bacteria, since LAB are known to tolerate higher concentrations of ethanol (Gold *et al.*, 1992) than other bacteria such as certain Gram-negative species, which are more sensitive (Ingram, 1976). This could explain why the ethanol production was high in the untreated flours even though electron acceptors were still present. In heat-treated flours the main source of energy appeared to be maltose and sucrose, since only low concentrations of glucose were detected in the unfermented flours. Since similar concentrations of succinate and mannitol were produced in both heat-treated and untreated flour, it is likely that the main electron acceptors were fumarate, malate and fructose. Lower concentrations of ethanol, lactate and acetate in the heat-treated compared with the untreated flour suggest that lack of monosaccharides (glucose and maltose) and not lack of electron acceptors (fructose, fumarate and malate) is the limiting factor for growth. This could explain the lower growth and higher pH seen in heat-treated flour fermented by *L. reuteri*. The higher growth and lower pH in heat-treated flour fermented by *L. plantarum* correlated to higher production of lactate. This indicates that *L. plantarum* can degrade polysaccharides or oligosaccharides to fermentable sugars. Differences were also observed in the metabolism of the *L. reuteri* strains, particularly in the production of succinate. One of the human probiotic strains (DSM 17938) had significantly lower production of succinate in both untreated and heat-treated flours of all barley varieties. These results could suggest that DSM 17938 did not use fumarate and malate as a source of electron acceptors, leading to the production of succinate. However, the results from the NMR analysis did not reveal any differences in the concentrations of malate and fumarate compared with the samples fermented by the other strains. The lower concentrations of succinate and similar concentrations of malate and fumarate could therefore indicate that the two latter compounds are used for the production of compounds other than succinate. Other species of lactobacilli are known to degrade malate and fumarate to lactate using malolactic enzymes (Stolz *et al.*, 1995), but this has never been detected in *L. reuteri*. Further

analysis of this is needed before any conclusions can be drawn regarding the presence and activity of such enzymes in this strain.

For most *L. reuteri* strains, the difference in concentrations of sugars could partially explain the difference seen in growth on each of the barley varieties. However, even though the concentrations of detected sugars were lower in some varieties compared with others, the growth was still higher. In an attempt to understand the relationship between growth of each individual strain and available sugars, sources of electron acceptors and amino acids, the results from the growth analysis was compared with the concentration of these compounds in the unfermented samples (Table 1). This revealed correlations between growth and sugar content and amino acid content, at least when comparing the heat-treated barley varieties with the lowest growth of *L. reuteri* to the varieties with the highest growth. As an example, DSM 17938 had the highest growth density in barley variety 224 and the lowest growth density in barley variety 120 and 249. Unfermented variety 224 had the highest concentrations of monosaccharides and disaccharides, as well as the second highest concentrations of amino acids, while 120 and 249 had the lowest concentrations of both groups of compounds. There was not a perfect correlation between sugar content, amino acid content and growth density, however, as seen *e.g*. in the growth of DSM 17938 in heat-treated flour. The second highest growth density was seen in barley variety 155, which had lower concentrations of both total sugar and total amino acid content than barley varieties 181 and 224, both of which had significantly lower growth density of DSM 17938. The ability of some *L. reuteri* strains to use sources other than available monosaccharides and disaccharides for energy could explain the results seen for *e.g*. barley variety 155 fermented by DSM 17938. Polysaccharide- or oligosaccharide-degrading enzymes with the ability to hydrolyse the complex sugars present in higher amounts in some of the barley varieties could be present and active in some *L. reuteri* strains and not in others. Such strain-specific enzymes have been observed in other studies (Saulnier *et al.*, 2011).

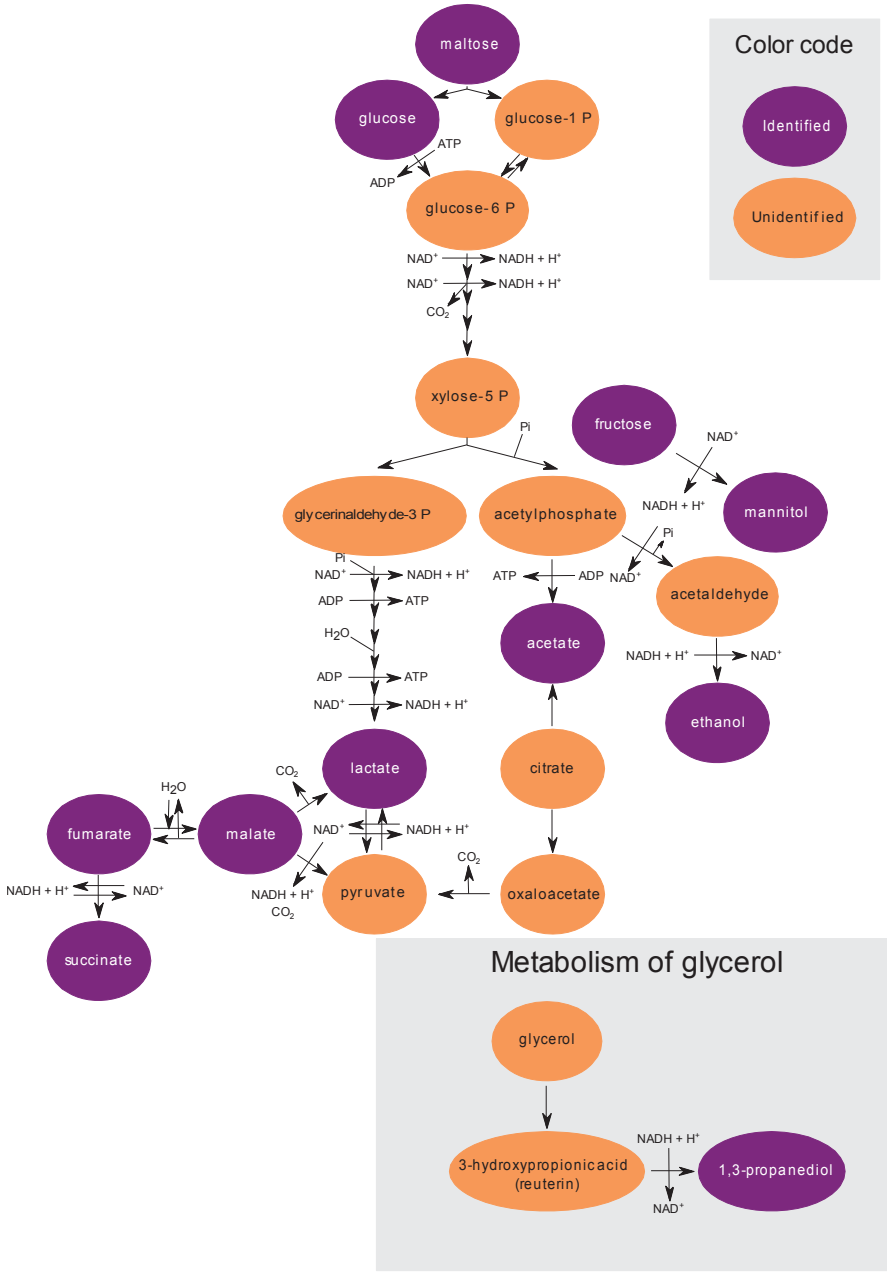


*Figure 5.* Metabolic pathways of *L. reuteri* during the fermentation of barley flour, based on previously published information (Stolz *et al.*, 1995). Compounds are colour-coded according to their presence in samples analysed in this study and are marked as either identified (purple) or unidentified (orange) based on the results from the 1H-NMR analysis. Most compounds were found in all samples with a few exceptions, *e.g.* 1,3-propanediol was only found in flour fermented with DSM 17938 and ATCC PTA 6475.

### 3.4.2 Potentially bioactive compounds in fermented barley

In addition to analysis of the normal metabolites produced during fermentation of barley flour by the different bacterial strains, differences in the production of possible bioactive compounds were studied. During a literature search, a few interesting bioactive compounds which can be produced by *L. reuteri* during the fermentation of different substrates were discovered. The plausible presence of these metabolites was targeted in the analysis for bioactive compounds, in addition to the search for novel metabolites. Through the use of 1H-NMR and the different online compound libraries, some of these bioactive compounds were identified in the samples. One such compound was GABA, which was found in all samples (untreated, heat-treated and unfermented), suggesting that it was present in the flour from the start. An increase in the concentration of GABA was seen in some fermented samples compared with unfermented, while other fermented samples showed a decrease in the concentrations. The increase in concentration was low and no correlations were found between increased GABA and use of a specific strain of bacteria. This indicates that there was no or only minor production of GABA during the fermentation of the different barley varieties. However, there were significant differences between barley varieties and between flour treatments. In heat-treated flour, the concentrations of GABA decreased by more than 60%. These observations suggest that the variety and treatment of the flour are important for the initial content of GABA in barley. The low or no production of GABA in these samples could have been due to the short incubation time, as previous studies have shown that the production of GABA increases over time for up to 96 hours (Stromeck *et al.*, 2011). According to the same study the production of GABA could be strain-specific, but no conclusion can be drawn about the strains used in this thesis until further experiments have been performed.

No other bioactive compounds were found using the chemical analysis methods applied in Paper I. However, one compound was identified in samples fermented by the two human probiotic strains, which could indicate the formation of yet another bioactive compound. In all barley varieties fermented by these two strains, 1,3-propanediol was detected. As explained in the introduction, some strains of *L. reuteri* are able to produce the antimicrobial substance reuterin by fermentation of glycerol. Since reuterin can be further reduced into 1,3-propanediol, the presence of this compound indicates that reuterin has been produced. The NMR-spectra for 3-hydroxypropionaldehyde (reuterin) is not included in any of the databases or libraries of compounds used in Paper I, making identification of the actual bioactive substance impossible (for now) using 1H-NMR. However, since both of these strains of *L. reuteri* tested positive for the presence of the gene and for actual production

of reuterin, assuming that reuterin was produced could be plausible even though only 1,3-propanediol was detected. Additional chemical analyses are needed to determine the concentration of reuterin produced during fermentation. Another indication of production of reuterin could be consumption of glycerol. Unfortunately, identification of glycerol was difficult or near impossible using 1H-NMR, since the spectrum for glycerol is located within a large cluster of other compounds, making it difficult to isolate from these compounds.

## 3.5 Evaluation of fermented barley in a cell model (Paper II)

In order to select a potential health promoting synbiotic formulation, it is important to evaluate that formulation using different models. Before proceeding to more expensive experiments such as animal models and clinical studies, it is important to establish that the formulation could actually have an effect using *e.g.* an *in vitro* model. In Paper II, the synbiotic formulations were evaluated using an epithelial cell model with the small intestinal cell line IPEC-J2.

### 3.5.1 Intestinal permeability in an intestinal epithelial cell model

IPEC-J2 is a non-transformed epithelial cell line isolated from the mid-jejunum of a neonatal piglet (Berschneider, 1989). This cell line has been widely used to study and characterise the epithelial cell interactions with pathogenic bacteria and viruses, especially focusing on the effect of pathogens such as *Escherichia coli* and *Salmonella enterica* (Brosnahan & Brown, 2012). The IPEC-J2 cell line has also been used for determining the adhesion of probiotics to cells and their ability to inhibit pathogen-induced inflammatory responses. In Paper II, the cell line was used for evaluating the effect of synbiotic formulations on the permeability of the cell layer and their protection against the pathogen enterotoxigenic *E. coli* (ETEC). As mentioned in the introduction, *L. reuteri* has been shown to inhibit the disruptive effect of ETEC on cell layer permeability (Liu, 2013). In order to determine whether metabolites produced by *L. reuteri* during the fermentation of barley have an effect against increased intestinal permeability, IPEC-J2 cells were pre-treated with the supernatant used in the chemical analysis. Pre-treated cells were then challenged with ETEC and the permeability was measured by transepithelial electric resistance (TEER) and by measuring the leakage of the fluorescent probe fluorescein isothiocyanate-dextran (FITC-dextran, 4kDa).

Early experiments on the IPEC-J2 cells revealed that supernatant from both fermented and unfermented barley flour caused an increase in the permeability

of the cell layer by itself (Figure 1 & 2 in Paper II). This increase in permeability was immediate and no recovery of the permeability was seen after 24 hours. Treatment with supernatant from heat-treated flour, on the other hand, led to a small decrease in permeability after a few hours, but complete recovery was achieved within six hours of treatment. Since the use of untreated flours itself caused an increase in permeability, these samples were not evaluated for their protective effect against ETEC. Further experiments were instead performed on samples from heat-treated flour and the effects compared with those of live probiotic bacteria.

In a final sub-experiment, a few selected heat-treated barley flour samples fermented by the two human probiotic strains were evaluated for their inhibition against the disruptive effect of ETEC on the permeability of the cell layer of IPEC-J2 cells. Pre-treatments were performed in triplicate and the effect of the synbiotic metabolites was compared to that of live bacteria using the same two human *L. reuteri* strains. After six hours of pre-treatment, the cells were challenged with ETEC for another six hours with TEER measured every other hour and a sample for the FITC-dextran assay was taken after six hours. The results showed that the pre-treatments did not have any protective effect against the disruption of cell layer permeability caused by ETEC in terms of TEER (Figure 3 in Paper II). The results from the FITC-dextran assay, on the other hand, showed a significant decrease in leakage in all but one pre-treatment compared with the untreated cells challenged with ETEC (Figure 4 in Paper II). Promising as these results are, they are only preliminary because of some problems with major variations in leakage seen in the untreated cells used as a control. Further experiments with fully functional controls are needed to confirm these observations.

# 4 Summary

The main findings in this thesis can be summarised as follows:

- *Lactobacillus reuteri* was successfully detected in, and isolated from, sourdoughs made from German rye flour and Chinese buckwheat and millet flour. This confirms the hypothesis that *L. reuteri* can be a frequent member of sourdoughs fermented at higher temperatures. However, *L. reuteri* was not detected in, or isolated from, any of the Swedish flours tested.

- Characterisation of the isolated strains of *L. reuteri* by various methods gave similar conclusions as previous studies on the origin of these sourdough strains. According to the results in this thesis, the most likely origin of the strains is contamination by rodent faeces.

- The fact that *L. reuteri* can grow well in cereal substrates was confirmed using six barley varieties with slightly different fibre composition and content. Bacterial counts ranged from 7.3-9.7 $\log_{10}$ CFU/g, which was similar to previous published values. The pH of barley fermented by *L. reuteri* reached similar or even lower values (3.4-5.3) compared with previous studies. Both strain- and species-specific differences in growth and pH reduction were observed, the most prominent being that *L. plantarum* 36E was able to grow better, and lower the pH more, than *L. reuteri* in heat-treated flour. Differences between *L. reuteri* strains were also mainly seen in heat-treated flour. DSM 17938 reached significantly higher growth density in two of the barley varieties, while two varieties gave significantly lower growth than all the other varieties. The growth density of the other strains, on the other hand, reached similar levels regardless of which barley variety they fermented. The differences between the two varieties with the highest growth of DSM 17938 and the two with the lowest can partly be

explained by the concentrations of available monosaccharides, disaccharides and amino acids. The results for DSM 17938 suggest that this strain is more dependent on easily available nutrients than the other strains for growth within 24 hours.

- Analysing the metabolism of *L. reuteri* and *L. plantarum* in different barley varieties using $^{1}$H-NMR revealed species- and strain-specific differences and similarities. One of the more noticeable differences between species of bacteria was that *L. plantarum* produced higher concentrations of lactate in heat-treated flours, suggesting that it could utilise carbohydrates present in barley that were not available to *L. reuteri*. This production of lactate was correlated with higher growth and lower pH in the samples fermented by *L. plantarum*. Strain-specific differences observed included *e.g.* a significantly lower concentration of succinate produced by one of the human probiotic strains (DSM 17938) in all barley varieties and in both flour treatments. Samples fermented with ATCC PTA 6475, on the other hand, contained lower concentrations of lactate and ethanol than all other *L. reuteri* strains in untreated flour. In heat-treated flour, the same differences in concentrations of lactate and ethanol were observed but they were not significant.

- The search for bioactive compounds produced by *L. reuteri* during the fermentation of barley flours using the methods available in this thesis only led to possible evidence of the production of reuterin. This evidence, in the form of production of 1,3-propanediol, was only detected in samples fermented with the two probiotic strains of human origin, ATCC PTA 6475 and DSM 17938. One other bioactive compound (GABA) was found to be present, but no significant production of this compound was detected by *L. reuteri* in the fermented barley flours. Since the production of GABA has been described for *L. reuteri* in previous studies, the low or no production seen in this thesis could be due to the shorter fermentation time used here.

- Evaluation of synbiotic formulations of barley and *L. reuteri* using an intestinal epithelial cell model (IPEC-J2) revealed that untreated barley flour increased the permeability of the cell layer, while heat-treated flour did not. Pre-treatment of the intestinal cells with different synbiotic formulations and live probiotic bacteria before a challenge with ETEC revealed that some of these pre-treatments had a potentially protective effect against the leakage of large molecules (FITC-dextran). However, none of the pre-treatments gave a protective effect against increased cell permeability as measured by TEER.

# 5 Future perspectives

Based on the results presented in this thesis, further research is needed in the following areas:

*Metabolic profiles of fermented foods*

The software used for the identification of compounds in 1H-NMR has its limitations, since it only contains the spectra of a total of 300 compounds. While the use of internet metabolome databases provides additional thousands of metabolites for the identification of metabolites, it does not help in the quantification of these metabolites. Parts of the spectra in this thesis were also left unidentified because of clustering of compounds, which made it impossible to single out individual compounds. Additional methods for the analysis of metabolites might be needed to provide a complete metabolic profile of each sample. Methods such as 2D-NMR and LC-MS could help achieve a more complete picture of the metabolic profiles. Use of these combinations of methods has been tested and proposed in other studies (Pan & Raftery, 2007).

*Production of bioactive compounds*

Since only a few bioactive compounds could be detected using the chemical analysis used in this study, different methods for analysis might be needed. For example, HPLC can be successfully used in detecting the production or release of phenolic acids (Hole *et al.*, 2012). Other methods including GC-MS and LC-MS have also been used for the detection of possible bioactive compounds in fermented cereals, with good results (Gorzolka *et al.*, 2012; Hu *et al.*, 2011). These are methods that could be used in combination with 1H-NMR for better coverage when screening combinations of cereal flour and probiotic bacteria for the production of possible bioactive compounds. Prolongation of the fermentation time to 48 or even 96 hours might also be necessary, since it has been shown that the production of bioactive compounds such as GABA

increases with longer fermentation times (Stromeck *et al.*, 2011). In order to find better synbiotic combinations, additional cereals or sub-cereals could also be analysed for their presence of bioactive compounds, since an increase in production has been seen in fermentation of *e.g.* sorghum (Coda *et al.*, 2010Coda *et al.*, 2010).

*Evaluation of synbiotic formulations in intestinal cell models*

The results from the experiments using the IPEC-J2 model are preliminary and additional experiments are needed before any conclusions can be drawn about the effect on intestinal permeability. Because of the disruptive effect of the fermented and unfermented, untreated barley flour, one could argue that the choice of cereal might be important for this kind of model. In order to exclude possible negative effects on cell membrane integrity caused by *e.g.* cereal proteins such as gliadins or glutenins, a comparison to cereals or sub-cereals lacking these proteins could be made, using *e.g.* fermented sorghum or buckwheat flour. The lack of effect on TEER when treating the cells with fermented, heat-treated barley flour could be due to the lower concentrations of bioactive compounds in these samples compared with the untreated barley flours. An increase in these concentrations might be possible with longer fermentation times, as indicated in the previous section. Comparisons of fermented barley flours with different fermentation times in the cell model might be necessary. In addition to comparing different cereals, the use of another type of cell line might be necessary. IPEC-J2 is a small intestine cell line and since many gastrointestinal disorders also affect the large intestine, the use of a large intestine cell line such as Caco-2 has been suggested in order to fully evaluate the effect of these synbiotic formulations.

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Zhou, Q., Zhang, B. & Nicholas Verne, G. (2009). Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome. *Pain,* 146(1), pp. 41-46.

# Acknowledgements

The work of this thesis was part of a thematic research program called BarleyFunFood which is a collaboration between the Faculty of Natural Resources and Agricultural Sciences at SLU and the industrial sector (Lantmännen, Biogaia and Lyckeby). The founding of BarleyFunFood was divided equally between the faculty and the industry. BioGaia has been the industrial partner for the work presented in this thesis.

First of all I would like to thank our little reuteri-group:

**Stefan**, tack för att du varit en sådan bra huvudhandledare trots ett fullspäckat schema. Jag kan fortfarande inte förstå hur du lyckas hålla alla de där detaljerna i huvudet, bland annat alla de olika namnen på stammar. Jag måste också tacka för att du och Hasse gav mig chansen att testa på att jobba inom den akademiska världen om än kanske bara för ett litet tag. Jag hoppas dock att vi får fortsätta att jobba tillsammans ett tag till så att vi kan slutföra vårt projekt.

**Hasse**, tack för att du stod ut med att jag störde dig titt som tätt med alla mina frågor angående allt mellan himmel och jord. Tack också för all hjälp i labbet, alla uppmuntrande ord och alla filmtips.

**Shokoufeh**, thanks for all the helpful work related discussions and friendly chat in our shared office. Hope I wasn't too unfriendly during the last stressful months☺.

And thank you all for a memorable first scientific conference in Holland, I'm really happy that the entire group could join.

Thanks to all the present and former colleagues at the Department of Microbiology for making it a very friendly and exciting workplace:

A special thanks to **German**, **Henrietta**, **Jule**, **Lea**, **Salome**, **Simon**, **Tina** and **Tong** for making my time at the department even more enjoyable, thanks for all the dinners, visits at pubs and sports bars (even though you weren't always interested in sports ☺), concerts and for joining me for climbing, tennis, badminton, squash or whichever sport or exercise.

Jag måste också tacka alla er nere på Institutionen för livsmedelsvetenskap: **Gunnel, Kristine**, **Roger** och **Susanne** för all hjälp med malningen av kornmjöl och för att ni tog er tid att svara på alla mina frågor.

Tack också till **Andreas**, **Anna-Greta**, **Haoyu** och **Torbjörn** på Institutionen för husdjurens utfodring och vård, för all hjälp med planeringen och utförandet av mina cellförsök.

Till sist men absolut inte minst vill jag tacka familjen för all support och uppmuntrande besök som gav mig nödvändiga pauser från livet i min egna lilla bubbla under stressiga perioder.