Genomic and phenotypic comparison of *Lactobacillus reuteri* isolates from food and intestinal ecosystems provides insights to probiotic applications

by

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Abstract

Some of the food fermenting lactobacilli are derived from the animal intestine. *Lactobacillus reuteri* provides a model organism to study molecular mechanisms of ecological adaptation from gut to food. The overall objective of this dissertation was to characterize ecological fitness of *L. reuteri* in food and intestinal ecosystems to guide the direction of probiotic applications.

The first goal of this dissertation was to assess carbohydrate transport and metabolism of *L. reuteri* strains to identify key metabolic traits specific to the cereal ecosystems. The genome-wide analysis indicates that food and intestinal isolates of *L. reuteri* do not differ with respect to the number and type of carbohydrate active enzymes. *In silico* transporter prediction, gene expression experiments, and phenotypic assays demonstrate that *L. reuteri* is characterized by the absence of ABC and PTS transporters, efficient utilization of cereals-associated oligosaccharides, and lack of glucose catabolite repression. This study provides experimental evidence to explain the co-existence of heterofermentative and homofermentative lactobacilli in a shared niche.

The second goal of this dissertation was to identify genetic signatures marking the adaption of cereal strains of *L. reuteri* from intestinal ancestors. Core- and pan-genome analysis on 16 *L. reuteri* strains demonstrates that sourdough-specific genes do not exist. Positively selected genes in sourdough isolates, however, are enriched in three functional groups, energy conversion, carbohydrate metabolism and defense systems while in gut ecosystem positively selected genes are significantly enriched in the function of translation. This suggests that sourdough and intestinal ecosystems differ in selection pressure. Competition in rye sourdough demonstrates that sourdough strains have higher or equal ecological fitness compared to rodent strains.

The third goal of this dissertation was to assess the ecological fitness of cereal strains of *L. reuteri* in the gastrointestinal tract of piglets. Host-adapted *L. reuteri* exhibits better persistence in the gastrointestinal tract of piglets compared to nomadic and free-living lactobacilli. The impact of probiotic lactobacilli on autochthonous lactobacilli was minor, but the antimicrobial reutericyclin produced by *L. reuteri* exerted a significant effect on autochthonous *Lactobacillus* communities.

This dissertation provides a basic understanding of the adaptation of intestinal *L. reuteri* to the food ecosystem, from evolution, ecology and metabolism perspectives. Such knowledge may improve current screening strategy for promising probiotics and starter cultures.

Preface

This thesis is an original work by Xin Zhao, which is according to the FGSR minimum thesis formatting requirements.

Chapter 2 is a literature review and is in preparation for submission to the journal, *Nutrients*. My contribution included searching literature, collecting information about *in vitro* and i*n vivo* acid resistance of enteric pathogens and probiotics, and writing the manuscript. Dr. Gänzle provided comments and further revised it.

Chapter 3 has been published as Xin Zhao and Michael G. Gänzle (2018), "Genetic and phenotypic analysis of carbohydrate metabolism and transport in *Lactobacillus reuteri*". *International Journal of Food Microbiology*, 272: 12-21. The study was designed by Dr. Gänzle and me. I conducted and analyzed all the experiments and wrote the manuscript together with Dr. Gänzle.

Chapter 4 has been published as Jinshui Zheng, Xin Zhao, Xiaoxi B. Lin and Michael G. Gänzle (2015), "Comparative genomics *Lactobacillus reuteri* from sourdough reveals adaptation of an intestinal symbiont to food fermentations", *Scientific Reports,* 5, 18234. Dr. Jinshui Zheng and I are the co-first authors of this publication. Dr. Jinshui Zheng performed bioinformatic analyses; Dr. Xiaoxi B. Lin performed competition experiments with reutericyclin-negative k.o. mutant strains; I performed other competition experiments and culture-dependent and independent analyses of sourdoughs. Dr. Gänzle, Dr. Zheng and me contributed to manuscript writing. All authors reviewed and approved the final version of the manuscript.

Chapter 5 has been submitted as Xin Zhao, Weilan Wang, Alysson Blaine, Sarah Miyata-Kane, Ruurd T. Zijlstra and Michael G. Gänzle, "Impact of allochthonous probiotic *Lactobacillus* spp.

on autochthonous lactobacilli communities in weaned piglets". Dr. Ruurd T. Zijlstra was in charge of the animal experiment. Weilan analyzed all the 16S rDNA pyrosequencing data. Weilan, Alysson, and Sarah designed strain-specific primers for probiotic strains employed in this study. Weilan was in charge of the collection of intestinal and fecal samples, and isolated DNA from all the intestinal samples. I prepared fermented feeds, and contributed to sampling and processing of samples. The corresponding manuscript was drafted by me. Drs. Ruurd T. Zijlstra, Gänzle contributed to the hypothesis development, experimental design, manuscript composition, and revision.

Dedication

This Ph.D. dissertation is dedicated to the Lord Jesus Christ and beloved families.

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CHAPTER 1 Introduction and objectives

1.1 Introduction

Food microbiota are partially derived from animal intestine. A 16S rDNA-based survey performed on various meat and seafood products indicated that a large proportion of contaminating microbiota on the selected fresh foods share high similarity with the normal microorganisms autochthonous to the gastrointestinal tract of animals, such as *Lactobacillus*, *Enterococcus*, and *Clostridium* (Chaillou et al., 2015). In addition to animal foods, fruits and vegetables harbor commensal bacteria or pathogens of the animal origin, typically contaminated with manure as a fertilizer or in irrigated water (Beuchat, 2002).

The presence of zoonotic pathogens, as well as fecal indicator bacteria in foods and water, firmly establishes the link between the dietary and intestinal microbiota. Zoonotic pathogens account for about 60% of all known pathogens to humans (Taylor et al., 2001). Foods and drinking water contribute to the transmission of various zoonotic pathogens from animals to humans (Hald et al., 2016). A multilocus sequence typing (MLST) based study demonstrated that outbreak strains of Shiga toxin-producing *E. coli* strains are highly related to the *E. coli* strains occurring in the animal food supply chain (Palanisamy et al., 2017). Gormley and colleagues (2008) proved that retail chicken is associated with *Campylobacter* emerging in humans. In addition, the whole genome sequencing approaches reveal that animal food is a common vehicle for antibiotic-resistant bacteria or genes entering the human population from livestock production (Koch et al., 2017). Many studies on detection of fecal indicator bacteria in water showed that animal feces leads to the contamination of groundwater and foods, particularly in rural areas (Ercumen et al., 2017; Field and Samadpour, 2007; Lamendella et al., 2013).

The association between food and intestinal microbiota is only beginning to emerge for food-fermenting lactobacilli and probiotic organisms. Some fermentation starter cultures, as well as lactobacilli isolated from spontaneous food fermentations, are autochthonous to the animal intestine; examples include *Lactobacillus amylovorus*, *Lactobacillus salivarius*, *Lactobacillus pontis*, *Lactobacillus panis* and *Lactobacillus reuteri* (Duar et al., 2017b). Sourdough lactobacilli represent an example. *L. pontis*, *L. reuteri*, and *L. amylovorus* are often isolated from the type II sourdough fermentation (Stolz et al., 1995). Members of *L. reuteri*- and *Lactobacillus delbrueckii*-groups found in sourdoughs are associated with the mammalian gut (De Vuyst et al., 2014; Gänzle and Ripari, 2016; Zheng et al., 2015). The continuous propagation of sourdough over a long time selects for *Lactobacillus* species/ strains that are highly adapted to the sourdough ecosystem, after a single initial inoculum or fecal contamination of raw materials (Gänzle and Ripari, 2016; Su et al., 2012). Substrate availability and fermenting process factors exert the selection pressure on sourdough microbiota (Gänzle and Ripari, 2016; Gobbetti et al., 2016). From an ecological perspective, 'dispersal limitation' as well as 'selection' rather than 'speciation' govern the development of sourdough *Lactobacillus* communities (Gänzle and Ripari, 2016). An initial study based on the taxonomic classification indicated that *L. reuteri* strains isolated from type II sourdoughs have a rodent or human intestinal origin (Su et al., 2012). Sourdough fermentation has a history of 6000 years in human civilizations (Brandt, 2005) but type II sourdough has a history of only 50 years (Brandt, 2007). Relative to the timescale of bacterial evolution, the time that passed since humans have fermented cereals is insufficient for species speciation to occur (Gänzle and Ripari, 2016). For instance, the youngest host-specific lineage of *L. reuteri* from a poultry ancestor was estimated to have emerged about 60,000 years ago by *in silico* analysis (Duar

et al., 2017a). Thus, sourdough strains of *L. reuteri* had not sufficient time to evolve into a new phylogenetic lineage from their ancestral origin (Duar et al., 2017a; Gänzle and Ripari, 2016).

L. reuteri provides a model system to characterizing the mechanism underlying host-specific adaptation. *L. reuteri* occurs in the digestive tracts of various vertebrates (Walter et al., 2011; Wood, 2012) and fermented cereals, including sourdough (Gänzle and Vogel, 2003). Multiple locus sequence typing revealed that intestinal strains of *L. reuteri* have co-evolved with their respective vertebrate hosts, and form host-specific phylogenetic lineages (Walter et al., 2011). Cereal strains of *L. reuteri* belong to the human- and rodent-specific lineages (Su et al., 2012). So far, whether any difference between cereal strains and intestinal strains of *L. reuteri* exist, either genetically or physiologically, remains to be determined. Three major evolutionary processes drive the bacterial adaptation in natural environments : i) genome size redution, ii) horizontal gene transfer, iii) mutations (Douglas and Klaenhammer, 2010; Makarova et al., 2006; O'Sullivan et al., 2009). *L. reuteri* is fastidious, relying on the availability of readily fermentable sugars (Walter et al., 2011). Carbohydrate utilization determines ecological competitiveness of lactic acid bacteria in various niches.

Food microbiota are also transient members of gut microbiota and may exert health effects to hosts (Derrien and van Hylckama Vlieg, 2015; Marco et al., 2017). Fermentation-associated lactobacilli have probiotic potential to human and animal hosts. Some *Lactobacillus* strains often found in fermented foods are identical or phylogenetically closed to *Lactobacillus* strains used as probiotics, thus putatively exert similar health-promoting effects to human and animal hosts. The probiotic activities are largely species dependent (Marco et al., 2017). These *Lactobacillus* species with documented probiotic activities involved in food fermentation include *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus fermentum*, *Lactobacillus plantarum*,

Lactobacillus paracasei and *Lactobacillus casei* (Marco et al., 2017). The ingestion of food fermenting lactobacilli might alter composition or functions of microbiota autochthonous to the human and animal gut (Derrien and van Hylckama Vlieg, 2015). Moreover, food matrix protects fermentation-associated lactobacilli against stress encountered during the intestinal transit.

1.2 Objectives

This thesis research hypothesizes that cereal and rodent isolates of *L. reuteri* are distinguishable at the genomic level and physiological level. To test this hypothesis, the present work aims to achieve four objectives:

i. To establish a correlation between acid resistance and probiotic efficacy of lactobacilli;

ii. To characterize carbohydrate utilization and transport systems for cereal and rodent isolates of *L. reuteri* by genomic and functional analysis;

iii. To recognize driving forces of speciation of *L. reuteri* populations by comparing genomes of cereal and rodent strains of *L. reuteri*;

iv. To assess the competitiveness of cereal isolates of *L. reuteri* against resident *Lactobacillus* spp. in the swine intestine.

The mammalian stomach has a low pH; the stomach is the first barrier to ingested microorganisms including probiotics and enteric pathogens (Yang et al., 2013). Acid resistance of food-borne bacteria may thus be important for survival during gastric transit and persistence in intestinal ecosystems. **Chapter 2** provides a literature review to establish a correlation between acid resistance and probiotic efficacy.

Carbohydrate metabolism not only contributes to the adaptation of bacteria but also determines trophic relationships of microbiota autochthonous to intestinal ecosystems. Homo- and hetero-

fermentative lactic acid bacteria differ in the glucose metabolism pathway. In food and intestinal ecosystems homo- and hetero-fermentative lactobacilli co-exist (Tannock et al., 2012; Wu et al., 2012). **Chapter 3** aims to assess carbohydrate metabolism and transport for heterofermentative *L. reuteri* to provide a molecular explanation for co-existence of homo- and hetero-fermentative lactobacilli in food and intestinal ecosystems, and to identify differences between rodent isolates and sourdough isolates of *L. reuteri*.

MLST and functional analysis demonstrated that sourdough strains of *L. reuteri* have a rodent origin (Su et al., 2012c). Type II sourdoughs have a history of 50 years; even several decades of continuous propagation of sourdough does not allow for occurrence of a new species from the ancestral isolates. **Chapter 4** aims to compare genomes of sourdough and intestinal strains of *L. reuteri* to recognize driving forces of diversification of *L. reuteri* population, and to identify positive selection and accessory genes in sourdough and intestinal strains of *L. reuteri*.

It is suggested that food- or feed-derived lactobacilli persist in the intestinal tract. It remains unclear, however, whether intestinal strains of lactobacilli improved persistence in the vertebrate gut, and how feed-derived lactobacilli alter autochthonous *Lactobacillus* communities in the swine gastrointestinal tract has not been documented. In particular, reutericyclin production by *L. reuteri* may modulate composition of autochthonous *Lactobacillus* communities in the swine gastrointestinal tract. **Chapter 5** aims to compare the competitiveness of environmental and nomadic lactobacilli with that of intestinal lactobacilli in the swine intestine.

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CHAPTER 2 The role of acid resistance in virulence of enteric pathogens and probiotic efficacy of probiotics

2.1 Introduction

The stomach initiates food digestion and prevents ingested microorganisms from reaching the intestine (Yang et al., 2013). Gastric juice consists mainly of hydrogen chloride and proteases and forms the gastric pH. In vertebrates, gastric juice is an ecological barrier to ingested microorganisms (Beasley et al., 2015). Animal species (e.g. the turkey vulture or red-tailed hawk) that feed on closely related organisms have significantly higher gastric acidity compared to other animal species (e.g. the ox, horse, or sheep) that feed on distantly related organisms, suggesting that gastric acidity increases with the risk of exposure to pathogens (Beasley et al., 2015). In human, clinical data indicate that the gastric pH of humans is associated with alteration of gut microbiota. For instance, the proton pump inhibitors (PPI) directly increase gastric pH. Meta-analysis based on clinical data demonstrates that patients receiving PPI therapy are more prone to infection by particular enteric pathogens including *Salmonella*, *Vibrio cholera*, *Listeria monocytogenes* and *Escherichia coli* (Bavishi and DuPont, 2011; Smith, 2003). Moreover, gastric acidity influences the composition of the microbiota of the stomach (Yang et al., 2013), small intestine (Lo and Chan, 2013; Lombardo et al., 2010) and colon (Imhann et al., 2015). Microbiota derived from foods or the oral cavity complement the gut microbiota by temporarily colonizing the gastrointestinal tract (Dal Bello and Hertel, 2006; Derrien and van Hylckama Vlieg, 2015; Zhang et al., 2016). Food and water are significant carriers for enteric pathogens.

Intrinsic acid resistance is associated with high viability for enteric pathogens or probiotic bacteria during gastric transit, thus conferring negative or positive health implications to humans. In the

human stomach, gastric juice forms extremely acidic conditions (pH 1.5-2.5 at fasting) (Morrison and Preston, 2016). The ability to overcome gastric pH during intestinal transit is regarded as a virulent factor for enteric pathogens (Foster, 1999). The FAO definition for probiotics is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* are most commonly used as probiotic cultures (Kleerebezem and Vaughan, 2009). The trait of tolerance to low pH has been incorporated into selection criteria for probiotic candidates (Amund, 2016). Remarkably, probiotic lactobacilli and enteric pathogens share comparable acid resistance strategies although they exert opposite health impacts on hosts. The comparison between probiotics and enteric pathogens concerning acid resistance may give insights into additional probiotic activities.

In this review, we briefly summarize the acid resistance strategies well studied in two representatives: *E. coli* and *L. reuteri*. The role of specific acid resistance mechanisms in survival and persistence of enteric pathogens and probiotic bacteria *in vivo* is discussed in subsequent sections. For enteric pathogens, the correlation of acid resistance and infectious dose is evaluated at population level based on available outbreak data. For probiotic bacteria, we assess the association of *in vitro* acid resistance with *in vivo* probiotic efficacy with emphasis on cases of commercial probiotic cultures. In addition, we outlie the protective effectiveness of food matrix to both probiotic bacteria and enteric pathogens.

2.2 Proton pump inhibitors (PPIs) influence enteric infection and gut microbiota

PPIs are commonly used drugs for many gastroesophageal diseases. PPI treatments directly reduces gastric acidity, resulting in enhanced susceptibility to infection caused by certain enteric

pathogens (Bavishi and DuPont, 2011; Leonard et al., 2007). Enteric pathogens differ in acid resistance and virulence in response to PPIs. A systematic survey based on available clinical data indicates that PPI treatment leads to a higher incidence of infection by common foodborne pathogens *Salmonella* spp., *Vibrio cholera*, *E. coli* and *Listeria monocytogenes* (Bavishi and DuPont, 2011). In addition to PPI treatment, age may influence gastric acidity and influence susceptibility to enteric infections (Beasley et al., 2015). For instance, premature infants and the elderly, who both have higher gastric pH compared to healthy adults, are more prone to enteric infections (Hu et al., 2012). Therefore, low pH of the human stomach plays a crucial role in preventing ingested enteric pathogens.

Although no data available illustrates how PPI treatment influences probiotic efficacy, much evidence demonstrates that PPI treatment affects microbiota in both the upper and lower gastrointestinal tracts. In the human stomach niche, PPI treatment with omeprazole decreased gastric acidity and substantially increased the number of culturable gastric bacteria (Sharma et al., 1984). The increased number of bacteria in both the lumen and mucosa of the stomach was seen in patients treated with PPI for an extended period (Sanduleanu et al., 2001). In the small intestine, PPI treatment has been demonstrated to induce bacterial overgrowth (Lo and Chan, 2013; Lombardo et al., 2010). In the colon niche, PPI treatment leads both to decreased richness of and alteration to gut microbiota. Notably, the number of oral bacteria and opportunistic pathogens increased in people receiving PPI intervention (Imhann et al., 2015).

2.3 Acid resistance mechanisms and their function *in vivo*

2.3.1 Acid resistance mechanisms of *E. coli* and *L. reuteri*

Since early studies on the subject, researchers have distinguished between two types of responses that organisms have against acidic stress: 'acid resistance' and 'acid tolerance response' (Bearson et al., 1997; Lin et al., 1995). 'Acid resistance' refers to mechanisms that allow un-adapted bacteria to survive acid shock at extremely acidic pH (pH 2.0-2.5) (Foster, 2001); 'Acid tolerance response' refers to mechanisms induced by challenging at moderately acidic pH (pH 4.0-5.0) that allows bacteria to survive the subsequent challenge of extremely acidic pH (Foster, 2001). Essentially, 'acid resistance' and 'acid tolerance response' are mediated by common structural genes, but regulated differently. The regulation mechanisms have been updated and reviewed elsewhere (Aquino et al., 2017; Castanie-Cornet et al., 1999; De Biase and Lund, 2015; Foster, 2004; Kanjee and Houry, 2013; Lund et al., 2014). For gram-negative *E. coli*, the acid resistance and acid tolerance response mechanisms have been intensively investigated and reviewed (Audia et al., 2001; De Biase and Lund, 2015; Foster, 1999, 2004; Kanjee and Houry, 2013; Lund et al., 2014). Gram-positive *L. reuteri* is well documented concerning the molecular basis of the acid stress response (Krumbeck et al., 2016; Su et al., 2011; Teixeira et al., 2014; Wilson et al., 2014). The structural components of acid resistance systems in *E. coli* and *L. reuteri* are illustrated in Figure 2-1 below. The outer membrane and periplasm are the first targets of proton attack for gram-negative *E. coli* as they are in direct contact with external pH (Slonczewski et al., 2009). But *L. reuteri* neither have the outer membrane or the periplasm as a gram-positive bacterium. Overall, three types of acid resistance mechanisms are present in the cytoplasm of *E. coli* and *L. reuteri*, including proton consumption (amino acid decarboxylase), ammonia production (urease, glutamine deaminase, arginine deiminase and adenosine deaminase), and macromolecule repair

(cytoplasmic chaperons). Amino acid decarboxylase is a crucial contributor to cytoplasmic pH maintenance for both *E. coli* and *L. reuteri* (Foster, 2004; Kanjee and Houry, 2013; Su et al., 2011). They perform proton-consuming decarboxylation on specific amino acids, such as glutamate, lysine, arginine, and histidine (Lund et al., 2014). The function of these acid resistance systems depends on substrates as well as extracellular pH (De Biase and Lund, 2015; Teixeira et al., 2014). Given that free amino acids are abundant in the stomach, and fermented foods, decarboxylase systems are thought to be crucial to the survival of *E. coli* and *L. reuteri* in environmental niches. Because various amino acid decarboxylases are active at different optimum pH values, they contribute protection to host cells at a wide range of pH, from 2.5 to 6 (Lund et al., 2014). Ammonia released from amino acids via deiminase and deaminase systems consumes cytoplasmic proton to form NH_4^+, resulting in an increased internal pH. The acid shock will lead to accumulation of damaged cellular proteins. Periplasmic chaperones (HdeA and HdeB in *E. coli*) (Kanjee and Houry, 2013) and cytoplasmic protease (Clp complex in *L. reuteri*) (Wall et al., 2007) are suggested to restore or remove damaged proteins.

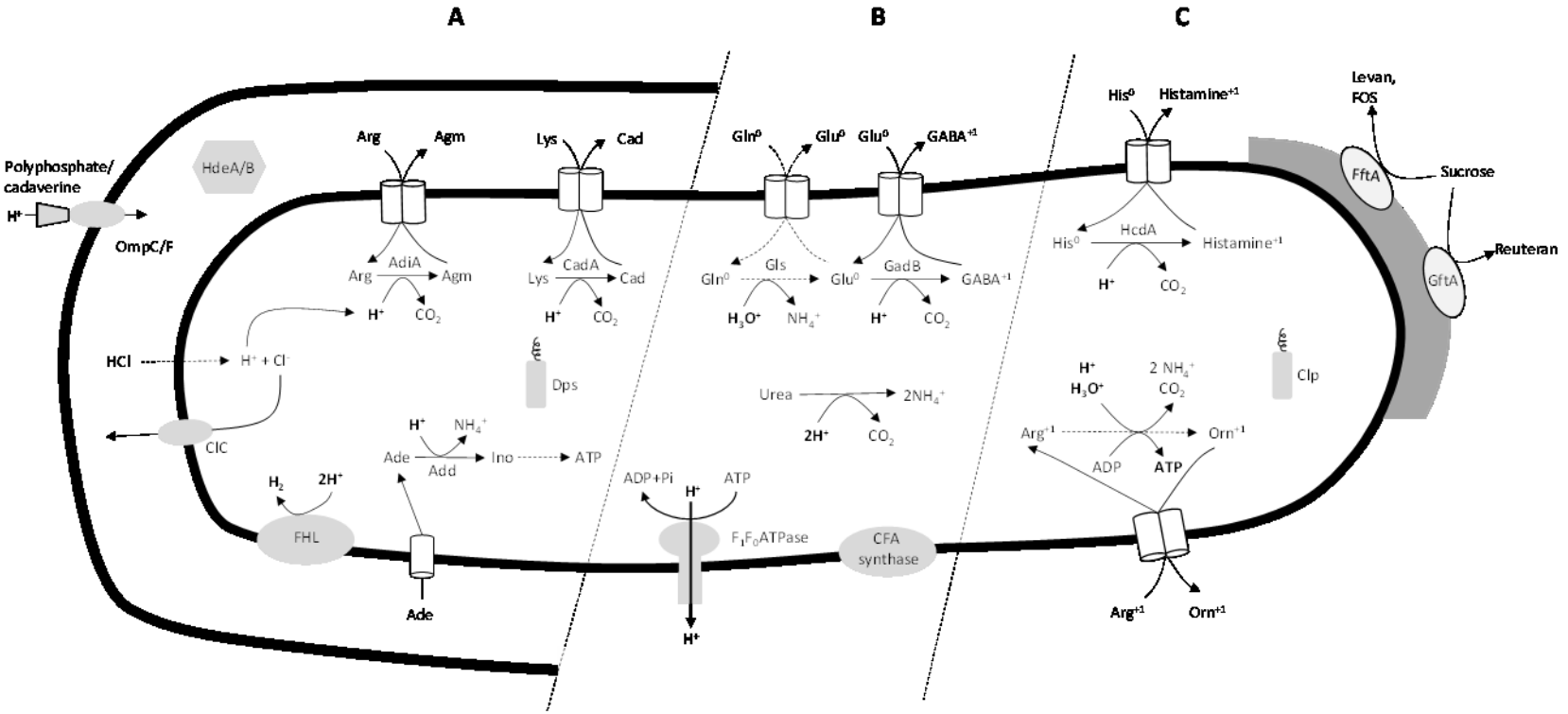

Figure 2-1 Overview of acid resistance mechanisms in *E. coli* and *L. reuteri*

Panel A: Acid resistance mechanisms only present in *E. coli*. Proton influx is reduced through polyphosphate or cadaverine blocking porins OmpC/F in the outer membrane (Delavega and Delcour, 1995). Periplasmic chaperones, such as HdeA and HdeB, protect periplasm and membrane proteins from proton attack (Hong et al., 2012). The formate hydrogen lyase (FHL) complex confers acid resistance under anaerobic conditions through consuming protons to generate H_2 (Kanjee and Houry, 2013; Noguchi et al., 2010). Arginine and lysine decarboxylase is only present in *E. coli*. Removal of positively charged protons by decarboxylase-dependent acid resistance systems lowers the internal pH but simultaneously leads to the accumulation of negatively charged chloride (dissociated from HCl) inside the cell, thereby resulting in potential hyperpolarization of the membrane. The CIC chloride channel in *E. coli* is demonstrated to act as a H+/Cl- antiporter to remove extra chloride ions from cells (Foster, 2004). The adenosine deaminase system has been recently recognized in *E. coli* and is suggested to contribute to the survival of *E. coli* under acidic conditions (Sun et al., 2012). Cytoplasmic Dps is suggested to restore or remove damaged proteins.

Panel B: Acid resistance mechanisms shared by *E. coli* and *L. reuteri*. The conversion of unsaturated fatty acids to cyclopropane fatty acids via CFA synthase reduces the permeability of the inner membrane, thereby proton influx across the membrane is decreased (Lund et al., 2014). Proton pump, F_1F_0ATPase is suggested to contribute to acid resistance. It directly expels cytoplasmic proton outside at the expense of ATP. Conversely, F_1F_0ATPase yields energy by translocating extracellular protons to the cytoplasm, supporting macromolecular repair mechanisms (Foster, 2004; Lund et al., 2014). Glutamate decarboxylase, as well as the glutamine deaminase system, is present in both *E. coli* (Lu et al., 2013) and *L. reuteri* (Teixeira et al., 2014). Glutamate converted from glutamine via glutamine deaminase is the substrate for subsequent glutamate decarboxylase. It is noted that the glutamine-based system is capable of working independently of the glutamate decarboxylase system (Lu et al., 2013; Teixeira et al., 2014). Urea degradation reaction generates ammonium ion by combining ammonia with internal protons. Both enterohemorrhagic *E. coli* and *L. reuteri* harbor the urea hydrolysis cluster. The urease enzyme has been demonstrated to enhance the survival of *E. coli* and *L. reuteri* during transit in the mouse stomach (Krumbeck et al., 2016; Steyert and Kaper, 2012).

Panel C: Acid resistance mechanisms only present in *L. reuteri*. Extracellular polysaccharides (reuteran and levan/FOS) converted from sucrose protect *L. reuteri* from external low pH, particularly at the stationary phase (Gänzle and Schwab, 2009; Kaditzky et al., 2008). Histidine decarboxylase is only present in *L. reuteri*. The arginine deiminase system is widespread in *Lactobacillus* spp. but absent from *E. coli*, which not only neutralizes cytoplasmic acidity but also provides ATP to F_1F_0ATPase to export cytoplasmic proton. The arginine deiminase system is active primarily at pH 3.5 in buffer (Lund et al., 2014; Teixeira et al., 2014). Cytoplasmic protease (Clp complex in *L. reuteri*) (Wall et al., 2007) are suggested to restore or remove damaged proteins.

2.3.2 Response of enteric pathogens and probiotics to acid stress *in vivo*

To date, acid resistance mechanisms for *E. coli* and *L. reuteri* have been well documented in the *in vitro* setting. However, fewer studies are available that assess acid resistance *in vivo*. Recent *in vivo* studies on acid stress response for *L. reuteri* and *E. coli* were summarized in Table 2-1 below. In a few *in vivo* studies, mice and cattle are used to address questions in this field. The GI tract of a mouse is thought to be a suitable model simulating that of a human's (Stevens and Hume, 1998). The mouse stomach consists of forestomach and corpus. Forestomach pH rises from 3.5 to 4.0 after diet consumption (Gärtner, 2001). The GI tract of cattle is the primary reservoir for enterohemorrhagic *E. coli* (EHEC) (Kaper et al., 2004). The use of appropriate animal models with omics- techniques allows for a better understanding of ecological roles of acid resistance mechanisms in the stomach niche (Armalyte et al., 2008; Price et al., 2004; Schwab et al., 2014; Wilson et al., 2014). Moreover, it may help us discover novel acid resistance mechanisms not recognized by *in vitro* or *in silico* methods (Hughes et al., 2010; Krumbeck et al., 2016).

2.3.2.1 *L. reuteri* in the mouse forestomach

The contribution of acid resistance mechanisms to the ecological competitiveness of organisms in part depends on specific niches. *L. reuteri* is an example. The putative acid resistance mechanisms in *L. reuteri* include D-alanylation of lipoteichoic acids, membrane composition alteration, the proton pump, amino-acid-dependent metabolisms and urea hydrolysis (Krumbeck et al., 2016; Teixeira et al., 2014; Wilson et al., 2014). These acid resistance mechanisms display a different

degree of effectiveness improving persistence of *L. reuteri* in hosts. *L. reuteri* colonizing the forestomach of mice is exposed to moderately acidic pH (pH 3-4). Many *in vitro* studies have suggested that glutamate decarboxylase systems are the most important contributor to acid resistance in bacteria (Diez-Gonzalez and Karaibrahimoglu, 2004). However, urease is demonstrated to predominantly contribute to the competitiveness of *L. reuteri* in rodent forestomach whereas glutamate decarboxylase system slightly improves its fitness (Krumbeck et al., 2016). *L. reuteri* strains adapted to the rodent forestomach can persist in type II sourdough (pH 3.2-3.6) (Su et al., 2012). Therefore, sourdough is a tractable model system to characterize acid resistance in mouse forestomach.

The contribution of acid resistance mechanisms to survival and persistence of organisms *in vivo* partly depends on environmental variables, such as extracellular pH (Teixeira et al., 2014) and substrate availability (Krumbeck et al., 2016; Su et al., 2011; Teixeira et al., 2014). It is noteworthy that activities of urease- and amino-acid-based acid resistance systems depend on the extracellular pH. Glutamate- and glutamine-based acid resistance systems of *L. reuteri* are primarily activated at pH 2.5 (Su et al., 2011; Teixeira et al., 2014), arginine deiminase pathway at pH 3.5 (Teixeira et al., 2014), and urease pathway at pH 4 (Krumbeck et al., 2016). Also, substrates for urease-, glutamate-, glutamine- and arginine-based systems are urea, glutamine, glutamate/ glutamine, and arginine, respectively. Substrates are essential for the function of amino-acid-dependent and urease systems (Krumbeck et al., 2016; Su et al., 2011; Teixeira et al., 2014). Urea is available in

the mammal's stomach but absent from cereal fermentations; free amino acids are derived from cereal protein hydrolysis during sourdough fermentation. This evidence may explain the different effectiveness of urease and amino-acid-dependent systems in different niches. Gastric pH results from hydrogen chloride, while sourdough acidity is mainly formed by weak acids (lactic and acetic acids) which are entirely disassociated inside cells and ultimately launch stronger proton attacks than HCl (Nguyen and Sperandio, 2012). In line with *in vitro* evidence (Teixeira et al., 2014), these acid resistance systems are hypothesized to work in a complementary but not overlapping manner *in vivo*.

2.3.2.2 Enterohemorrhagic *E. coli* in cattle rumen and the mouse gastrointestinal tract

E. coli possesses four genetically distinct systems mediating resistance to acidic conditions, including the glucose-repressed RpoS-dependent system, the glutamate decarboxylase system, the arginine deiminase system and the lysine decarboxylase system (Foster, 2004). EHEC as commensal in cattle can survive gastric transit and colonize the lower intestine of cattle. The glutamate decarboxylase system predominantly contributes to the survival and persistence of EHEC during gastric transit in cattle (Price et al., 2004), while the RpoS-dependent system is the predominant contributor to viability in apple cider (Price et al., 2000). The acyl-homoserine lactones (AHLs) in cattle via *sdiA* activate *gadBC* operon of EHEC and thus improve acid resistance before entry into the acidic stomach of cattle (Dziva et al., 2004; Hughes et al., 2010; Sheng et al., 2013). AHLs produced by bacteria are only present in the rumen and absent from

other compartments in cattle GI tract of cattle (Dziva et al., 2004; Hughes et al., 2010; Sheng et al., 2013). In the mouse model, the competition experiment between the wild type strain and urease-deficient mutant demonstrated that urease enzyme of EHEC strains plays a significant role in their survival in gastric transit and colonization in the gut (Steyert and Kaper, 2012).

2.3.2.3 Association of *in vitro* acid resistance and *in vivo* viability of probiotics

In vitro acid resistance doses not predict *in vivo* viability of probiotic strains. The commercial probiotic strain *L. paracasei* F19 completely loses viability when challenged with simulated gastric juice (pH 2.0) (Charteris et al., 1998). Conversely, its ability to survive upper intestine passage and transiently establish in the colon was seen in a human feeding trial (Crittenden et al., 2002) (Table 2-2 below). A similar controversy is seen with other commercial probiotics, including *L. gasseri* ATCC33323, *L. casei* LC1, *L. rhannosus* LR3, *L. plantarum* LP1, and *L. paracasei* IMPC2.1 (Table 2-2 below). Overall, they exhibit remarkable reduction (4-8 log) in *in vitro* acid resistance assays but display high fecal recovery in human trials. *In vitro* screening for acid resistance thus may exclude strains exhibiting good survival and probiotic efficacy *in vivo*. Mainville *et al*. (2005) identified divergence in acid resistance of probiotic bacteria assayed by two methods: the conventional method and the dynamic upper-intestine simulator that resembles conditions of food ingestion and digestion. Notably, some probiotic strains including the well documented probiotic *L. rhamnosus* GG that exhibited inferior survival in the medium of pH 2, exhibited improved survival in the dynamic model (Mainville et al., 2005). It is demonstrated that

two probiotic strains, *L. johnsonii* NCC533 and *L. paracasei* NCC2461, which share close acid resistance phenotypes and other *in vitro* features, colonize the mouse gut differently (Ibnou-Zekri et al., 2003).

Table 2-1 *In vivo* studies on acid stress response of *E. coli* and *L. reuteri*

Organism	Identification approach	Genes/ proteins responsible for acid resistance	Effect	Reference
L. reuteri autochthonous to mouse forestomach	Meta-transcriptome	Urease, glutaminase, glutamate decarboxylase, arginine deiminase and arginine/ ornithine antiporter, *dlt* operon, cyclopropane-fatty-acyl-phospholipid synthase	Acid resistance genes overexpressed in the mouse forestomach	(Schwab et al., 2014)
L. reuteri 100-23	Transcriptome + mutagenesis	*ureC* (Urease, α subunit), *gls3-gadB* operon (glutaminase and glutamate decarboxylase)	UreC and Gls3-GadB increase fitness in the mouse forestomach	(Wilson et al., 2014)
L. reuteri 100-23	Mutagenesis	*ureC* (Urease, α subunit), *gadBC* (glutamate decarboxylase)	UreC and gadBC increase fitness in the mouse forestoamch	(Krumbeck et al., 2016)
E. coli O157: H7	Mutagenesis	*rpoS* (glucose-repressed *rpoS*-dependent acid resistance)	RpoS increases fecal shedding of the viable wildtype strain inoculating-calves	(Price et al., 2000)
E. coli O157: H7	Mutagenesis	*gadC*	GadC increases fecal shedding of the viable wildtype strain inoculating calves	(Price et al., 2004)
EHEC strains	Mutagenesis + metagenomics	*sdiA* (regulator of QS, activates *gad* expression indirectly)	*sdiA* increases persistence and colonization of the wildtype strain in cattle rumen	(Hughes et al., 2010; Sheng et al., 2013)
EHEC strains	Mutagenesis	Urease locus	Urease contributes to the survival in the mouse stomach and colonization in the gut	(Steyert and Kaper, 2012)

Table 2-2 *In vitro* acid resistance phenotype and fecal recovery for commercial probiotics

Group	Species[a]	Acid challenging condition	*In vitro* survival	Fecal recovery[b]
L. delbrueckii group	*Lactobacillus johnsonii* La-1	pH2, 1h	90% (Aiba et al., 2015)	
	Lactobacillus gasseri ATCC33323	pH2, 2h	0% (Azcarate-Peril et al., 2008)	
L. salivarius group	*Lactobacillus acidophilus* LH5	pH1.5, 1h	＜0.001% (Larsen-Vefring, 2013)	
	Lactobacillus acidophilus LA5	pH3, 1h	45% (Gebara et al., 2013)	10^5 (Savard et al., 2011)
L. casei group	*Lactobacillus casei* LC1	pH1.5, 1h	0.1% (Guergoletto et al., 2010)	
	Lactobacillus casei Shirota	pH2.5, 2h	90% (Chan et al., 2010)	
	Lactobacillus rhamnosus LR3	pH1.5, 1h	＜0.001% (Larsen-Vefring, 2013)	
	Lactobacillus rhamnosus GG	pH2, 15min	＜0.001% (Mainville et al., 2005)	10^8 (Savard et al., 2011)
	Lactobacillus paracasei F19	pH3, 1.5h	5% (Charteris et al., 1998)	1.3×10^6 (Crittenden et al., 2002)
	Lactobacillus paracasei IMPC2.1	Simulated gastric acid	0.1% (Valerio et al., 2006)	10^7 (Valerio et al., 2006)
L. plantarum group	*Lactobacillus plantarum* LP1	pH1.5, 1h	＜0.001% (Larsen-Vefring, 2013)	
L. reuteri group	*Lactobacillus reuteri* DSM20016	pH3, 3h	76% (Jensen et al., 2012)	
	Lactobacillus reuteri DSM17938	pH3, 3h	26% (Jensen et al., 2012)	
	Lactobacillus fermentum CECT5716	Dynamic gastric simulator	30% (Martín et al., 2005)	
Bifidobacterium	*Bifidobacterium animalis* Bb12	Simulated gastric acid	90% (De Castro-Cislaghi et al., 2012)	8×10^7 (Larsen et al., 2006)

[a] Probiotic strains shown are all commercially used cultures.

[b] Fecal recovery data is obtained from human trials and shown in the table if available.

2.4 Link between acid resistance and infectious dose/ probiotic survival *in vivo*

2.4.1 Correlation between acid resistance with infectious dose of enteric pathogens

The infectious dose is referred to as the minimal amount of a pathogen required to cause illness to host (Leggett et al., 2012). Some enteric pathogens require a high infectious dose ($\geqslant 10^6$ CFU) including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), *Vibrio cholerae* and *Yersinia enterocolitica*. *L. monocytogenes* and *Salmonella* spp. both exhibit conditional infectious dose depending on host susceptibility; enterohaemorrhagic *E. coli* (EHEC) and *Shigella flexneri* require an exceptionally low infectious dose (Table 2-3 below). The stomach is considered a primary barrier against ingested enteric pathogens (Tennant et al., 2008). Hypochlorhydric mouse model demonstrated that reduction in gastric acidity results in substantially lower immunity to infection by *Yersinia*, *Salmonella* and *Citrobacter* (Tennant et al., 2008). Acid resistance contributes to the viability of ingested organisms during gastric transit, and is therefore thought to be a virulence factor for enteric pathogens. However, it has not yet been established whether the degree of acid resistance directly correlates with the infectious dose. This section outlined the association of acid resistance with the infectious dose based on laboratory, clinical and outbreak data.

2.4.1.1 Intrinsic acid resistance matters: *E. coli* and *Shigella*

The enterohemorrhagic *E. coli* (EHEC) and *Shigella* spp. show a uniquely low infectious dose among common enteric pathogens, 1-100 CFU and 10-200 CFU, respectively as reported in

foodborne outbreaks (Table 2-3 below). In buffer systems, most strains of *E. coli* and *Shigella* spp. are capable of surviving extreme acidity (pH 2.5 for 2-3 hours particularly in stationary phase), whereas *Salmonella* spp. and *V. cholera* are considerably inhibited on the same acidic condition (Lin et al., 1995). The bovine gastrointestinal tract is considered a natural reservoir for EHEC *E. coli*, and most foodborne outbreaks are associated with beef products (Hara-Kudo and Takatori, 2011; Nguyen and Sperandio, 2012; Tuttle et al., 1999).

2.4.1.2 Acid resistance is not the reason for hyperinfectivity: *C. rodentium,* and *V. cholerae*

Citrobacter rodentium, a mouse pathogen (Schauer et al., 1995) is characteristic of hyperinfectivity induced by passage through the mouse intestine, in which ID_{50} (ID_{50} indicates the dose required to kill 50% test population) of the ingested *C. rodentium* is significantly reduced to approximately 5.4 log in the mouse model (Smith and Bhagwat, 2013). *C. rodentium* demonstrates a convergent host infection strategy with human pathogens, EHEC and EPEC. Those three pathogens have a common type III secretion system to induce attaching and effacing (A/E) lesions essential for virulence (Petty et al., 2010). As *C. rodentium* is a natural mouse pathogen that is related to *E. coli*, it provides a good *in vivo* model for A/E lesion forming pathogens (Mundy et al., 2005).

Notably, such a phenotype of hyperinfectivity is not due to induction of acid resistance of the mouse-passaged *C. rodentium* (Smith and Bhagwat, 2013). Either the parental strain or mouse-adapted strain of *C. rodentium* is somewhat sensitive to low pH because they lack amino-acid-

dependent acid tolerance systems. Instead, the elevated expression of colonization factors after mouse passage may be a significant contributor to hyperinfectivity (Smith and Bhagwat, 2013).

Likewise, the temporary hyperinfection state is also observed in *Vibrio cholerae* O1 and O139 strains after passage through human or mouse (Alam et al., 2005; Merrell et al., 2002). *V. cholera*, known as marine bacteria, enters into the human host via food vehicles (Thompson et al., 2004). The infectious dose for *V. cholerae* ranges between 10^4 and 10^6 CFU typically when consumed in food (Table 2-3 below). Although *cadA* encoding lysine decarboxylase medicates acid tolerance response during human passage, rapid multiplication and improved colonization in the small intestine is primarily responsible for improving competitive fitness for human-adapted cells (Alam et al., 2005; Angelichio et al., 2004; Merrell and Camilli, 1999).

2.4.1.3 Correlation of acid resistance phenotype and outbreak data is not firm

Mutagenesis studies as stated above demonstrate that the knockout of particular acid resistance genes of enteric pathogens results in significant loss of their virulence potential. However, the correlation between acid resistance phenotype assessed *in vitro* and the outbreak data are not firmly determined in part due to the intraspecies variation in acid resistance and uncertainty of outbreak data.

EHEC appears to have a uniquely low infectious dose compared to other *E. coli*. O157: H7 serovar is a typical representative in EHEC. Whether *E. coli* O157: H7 strains have superior acid resistance to other *E. coli* is controversial. The multivariate analysis of stress resistance for 33

strains of EHEC *E. coli* O157: H7 illustrates that the strains associated with human outbreak cases appear more resistant to multiple environmental stresses including acid (Elhadidy and Álvarez-Ordóñez, 2016). Although most EHEC *E. coli* O157: H7 strains share low infectious doses, their ability to survive acidic stress is very diverse (Bergholz and Whittam, 2007; Kim et al., 2015). Collectively, these experimental results point out that EHEC *E. coli* O157: H7 strains are not exceptionally resistant to low pH, compared to other members of EHEC and even commensal *E. coli*.

The uncertainty of infectious dose data makes the establishment of a relationship between acid resistance and infectious dose complicated. Infectious dose data is typically collected from volunteer studies, counts of outbreak investigations, or a literature review on the infectious dose for a defined population in a particular food matrix. The minimal infectious dose is strain-dependent such that it has uncertainty (Schmid-Hempel and Frank, 2007). Additionally, the immune status of an individual human also plays a vital role in determining the observed infectious dose of specific pathogens. Different assessment methods may yield unique infectious dose data for given pathogens. For instance, the infectious dose determined by the US Food and Drug Administration is different from the one determined by Health Canada for *Salmonella entrica* and *Yersinia* spp., by two and five orders of magnitude, respectively (Schmid-Hempel and Frank, 2007).

2.4.2 Link of acid resistance with the effective dose of probiotics

Acid resistance is a principal determinant to the viability of ingested probiotic organisms during intestinal passage (Bezkorovainy, 2001), and thus it is incorporated into the selection criteria for probiotics (Saarela et al., 2000). *Lactobacillus* spp. and *Bifidobacterium* spp. are commonly used for probiotic application partially because they are commensal members in the mammalian gastrointestinal tract (Kleerebezem and Vaughan, 2009). *Lactobacillus* spp. naturally occur in the upper intestine of vertebrates, with a natural resistance to acidic pH as low as 3 (Duar et al., 2017; Jin et al., 1998), whereas *Bifidobacterium* spp. are mainly associated with human's colon and feces, exhibiting less resistant to the extremely acidic pH compared to lactobacilli (Dunne et al., 2001; Reuter, 2001). Most of the *Lactobacillus* strains commonly isolated from fecal samples are only temporary passengers in the human intestine. They originate from the oral cavity or food (Dal Bello and Hertel, 2006; Walter, 2008). *Lactobacillus* spp. autochthonous to the human intestine include *L. crispatus, L. gasseri*, *L. reuteri*, *L. ruminis* and *L. salivarius* (Dal Bello and Hertel, 2006). *Bifidobacterium breve*, *B. infantis*, *B. longugm* and *B. bifidum* are common species detected in the human intestine, particularly in newborn infants (Boesten et al., 2011; Satokari et al., 2001).

The minimum dose of probiotic cells required to confer the claimed health benefits to humans is known as an effective dose. Usually, probiotics are supplemented with food or diet at a dose ranging between 10^8 and 10^{12} CFU/ day (Ouwehand, 2017). One may assume that the acid

resistance phenotype in the *in vitro* assay of a probiotic strain is negatively proportional to its effective dose. However, whether *in vitro* acid resistance is correlated with probiotic performance (e.g., fecal recovery, effective dose) in humans is not fully determined. *In vitro* evaluation of acid resistance as well as a fecal recovery in human intervention is summarized in Table 2-2 above, above for commercial probiotics. The following section aims to unveil the link between *in vitro* acid resistance and *in vivo* survival based on available experimental data acquired from commercial probiotic organisms.

In vitro acid challenging conditions vary among literature. Inconsistency in testing conditions of the acid resistance phenotype is a barrier to making comparison across independent studies. In conventional assays, the pH used ranges from 1.5 to 4.5; acid treatment time used ranges from 30 to 240 min. Conventional assays were usually performed in the PBS acidified with concentrated HCl, or simulated gastric juice. Human gastric juice is proposed to be a better system for acid resistance phenotype assessment (Dunne et al., 2001). Normalizing test results based on a type strain is recommended.

Since the gastric pH of humans is not constant, the acid resistance phenotype assessed by traditional *in vitro* methods may not be an accurate indication of the ability of ingested bacteria to survive intestinal transit. Clinical data shows that gastric disorder and medical interventions influence stomach pH. Also, dietary consumption is another factor that alters baseline acidity of the human stomach (Tompkins et al., 2011). Foodstuff will extend empty time and temporarily

increase pH in the stomach (Simonian et al., 2005), resulting in the high survival of ingested bacteria in gastric transit. The dynamic gastric model has been developed by including a food matrix as part of the model that allows evaluating acid resistance in pH (Mainville et al., 2005).

Taken together, *in vitro* results of acid resistance assessment cannot be extrapolated to animals or human subjects. Administration of high doses of probiotics compensates for the loss in viability of probiotics during gastric transit. Thus, we argue that *in vitro* acid resistance assessment is not necessary for the selection criteria of probiotic candidates.

Table 2-3 Association of acid resistance phenotype and infectious dose for foodborne pathogens

Organism	Natural reservoir	Infectious dose in human (CFU)[a]	Acid challenging condition	*In vitro* survival
		Low infectious dose pathogens		
Enterohaemorrhagic *E. coli* (EHEC)	Ruminant (Bryan et al., 2015)	1-100	pH 3, 2h	80% (Lin et al., 1995)
Shigella flexneri	Humans and higher primates (Peterkin, 1993)	10-200 (Kothary and Babu, 2001)	pH 2.5, 2h	50% (Gorden and Small, 1993)
		Infectious dose dependent on host		
Listeria monocytogenes	Soil, water, silage, decaying vegetation, fecal material (Fenlon, 1999)	10^2-10^7	pH 3.5, 1h	30% (Cheng et al., 2015)
Salmonella Enteritidis	Hen housing environments, birds, eggs (Braden, 2006)	10-10^6	pH 3, 2h	＜0.001% (Gorden and Small, 1993)
		High infectious dose pathogens		
Vibrio cholera	Human, animals in aquatic environments (Kerr, 2004)	10^4-10^6 (Kothary and Babu, 2001)	pH 2.5, 2h	＜0.001% (Waterman and Small, 1998)
Yersinia enterocolitica	Pig, grazing farm animals (Le Guern et al., 2016)	10^8	pH 3, 2h	85% (De Koning-Ward and Robins-Browne, 1995)
Enterotoxigenic *E. coli* (ETEC)	Human, surface water (Gonzales-Siles and Sjöling, 2016)	10^8-10^{10}	pH 3, 2h	20% (Gorden and Small, 1993)

[a]Minimum infectious dose data is obtained from Pathogen Safety Data Sheets (https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment.html) unless indicated otherwise.

2.5 Protective effect of food matrix

A food matrix is the primary vehicle for delivering probiotic cultures to humans, while freeze-dried powder, fermented cultures, and capsules are the primary forms of probiotic delivery in animal trials. Food matrix provides a protective barrier with incorporated probiotic bacteria both in food products and during intestinal transit.

The ingested food influences gastric acidity and empty time. The foodstuff transiently elevates gastric pH. A clinical survey shows that consumption of a standard western meal increased the gastric pH of young and elderly groups to 6.6 and 6.2, respectively. The gastric pH at a fasting state for young individuals is 1.7 and 1.3 for elderly individuals (Dressman et al., 1990; Russell et al., 1993). Empty time is defined as the length of time food remains in the stomach, depending on chemical ingredients of the ingested food. Liquid food immediately passes into the small intestine after ingestion, while particle food has a lag period before leaving the stomach.

Specific food matrices are deliberately employed to protect probiotics from acid stress. The protective effect of food matrix might be strain- or species-dependent. Yogurt bacteria *Lactobacillus delrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* exhibit different viability under the protection of yogurt during intestinal transit (Elli et al., 2006). Johansson et al. (1993) evaluated colonization ability in the human intestinal mucosa of closely related *Lactobacillus* strains ingested with the fermented oatmeal soup. Strains either within the same species or from the same sources differed in performance in the human GI tract (Johansson et al., 1993). The dose of probiotic bacteria inoculated in food may influence the protective effect of particular food matrices. *B. animalis* strains exhibited no difference in fecal quantification when with a relatively high dose ($6\times10^{9} – 2\times10^{11}$ CFU/g) ingested with fermented milk or freeze-dried

powder (Rochet et al., 2007). In contrast, fecal recovery of the *L. plantarum* strain (Klingberg and Budde, 2006) and *L. rhamnosus* strain (Saxelin et al., 2010) is significantly enhanced by food matrices, when lower doses (6×10^9, 2×10^9 CFU/g, respectively) were inoculated.

Also, the food matrix influences the viability of contaminating enteric pathogens. *Salmonella* Typhimurium exhibits significantly higher survival at lethal acid conditions when inoculated into ground beef and boiled egg white (Waterman and Small, 1998). It is hypothesized that neutralization of acidity in the microenvironment of solid food at least partly contributes to a lower infectious dose of some acid-sensitive enteric pathogens (Waterman and Small, 1998). A study shows that the *E. coli* strain inoculated in meat as a solid form of food matrix, reaches ileac compartment with substantially fewer viable cells compared to the strains inoculated in milk as a liquid form of food, suggesting that solid foods result in the prolonged exposure of the cells to gastric acidity (Gänzle et al., 1999). Solid food and liquid food differs in the gastric transit dynamics. Solid foods are transported out of the stomach at a minimal rate during a lag phase of 20-30 min after entering the gastric compartment whereas liquid foods transit the stomach at an exponential rate immediately after entering to the stomach. It has been established that specific protein in food protects *Salmonella* Dublin from inactivation by acidity in a dynamic stomach simulator (Birk et al., 2012).

As described in previous sections, the glutamate decarboxylase-dependent system plays a dominant role in acid resistance of most pathogens and probiotics. Many food ingredients contain free glutamate, such as autolyzed yeast, malt extract, and whey protein, while some foods rich in protein have a high abundance of bound glutamate. The free glutamate in low-acid food is demonstrated to substantially contribute to the survival of *L. monocytogenes* (Cotter et al., 2001). Nowadays, the emerging and promising method, micro-encapsulation is hypothesized to protect

probiotic bacteria from stressful conditions both in food products and during gastrointestinal transit (De Prisco and Mauriello, 2016). Despite *in vitro* success of micro-encapsulation, confirmative *in vivo* evidence of its protective effect to probiotic bacteria is very limited. A recent study on sodium caseinate (SC), a milk-based matrix of micro-encapsulation reveals that good protective effectiveness of SC *in intro* does not translate into success *in vivo* (Würth et al., 2015).

2.6 Concluding remarks

E. coli and *L. reuteri* possess overlapping but different acid resistance systems. The contribution of acid resistance mechanisms to the viability and competitiveness of organism in specific niches depends on multiple factors, including external pH, substrate availability and signal molecules. The infectious dose is host- and strain- dependent. Acid resistance is associated with virulence in EHEC strains, whereas acid resistance is not associated with hyperinfectivity for human-passaged *C. rodentium* and *V. cholera*. Given that stomach pH is dynamic, *in vitro* acid resistance cannot accurately indicate *in vivo* survival of probiotics. Moreover, food matrix protects incorporated probiotics from acidic pH and thus improves survival of given probiotics even with poor acid resistance. Therefore, we argue that *in vitro* acid resistance assessment is not a necessary consideration for selection criteria of probiotics.

2.7 References

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CHAPTER 3 Genetic and phenotypic analysis of carbohydrate metabolism and transport in *Lactobacillus reuteri*

3.1 Introduction

Production of a majority of food fermentations involves lactobacilli as abundant members of fermentation microbiota, and the conversion of carbohydrates to lactic acid is a major contributor to the quality and safety of these fermented foods (Gänzle, 2015). Carbohydrate metabolism also provides the main source of metabolic energy in lactobacilli and thus contributes to their ecological fitness (Gänzle, 2015). Lactobacilli preferentially metabolize monosaccharides and oligosaccharides; enzymes for extracellular hydrolysis of polysaccharides are exceptional (Gänzle and Follador, 2012). The genus *Lactobacillus* includes a large and diverse number of species; physiological, ecological, and phylogenetic properties separate *Lactobacillus* spp. in two major clades comprising homofermentative and heterofermentative lactobacilli (Duar et al., 2017c; Zheng et al., 2015a). Homofermentative lactobacilli metabolize glucose by glycolysis; heterofermentative lactobacilli metabolize glucose by the phosphoketolase pathway; pentoses are metabolized by the phosphoketolase pathway or the pentose phosphate pathway by organisms in both groups (Gänzle, 2015; Zheng et al., 2015a). The regulation of carbohydrate metabolism differs between homofermentative and heterofermentative lactobacilli. Homofermentative lactobacilli preferentially metabolize glucose; the use of alternative carbon sources is generally repressed by carbon catabolite repression if glucose is available (Andersson et al., 2005; Gänzle et al., 2007; Monedero et al., 2008). Carbohydrate transport is mediated by members of the ATP-binding cassette (ABC) superfamily of ABC-transporters, secondary transporters of the Major Facilitator Superfamily (MFS), or phosphotransferase systems (PTS). PTS systems, which

mediate phosphorylation and transport of mono- and di-saccharides, directly or indirectly mediate carbon catabolite repression through interaction with the catabolite control protein A (Andersson et al., 2005; Galinier and Deutscher, 2017; Monedero et al., 2008). Genomes of heterofermentative lactobacilli code for fewer genes for PTS systems when compared to homofermentative lactobacilli (Zheng et al., 2015a). In contrast to homofermentative lactobacilli, glucose transport and metabolism is not constitutive but induced by the substrate in heterofermentative lactobacilli (Neubauer et al., 1994; Ye and Jr, 1995) and the utilization of maltose, sucrose, and pentoses is not repressed by glucose (Ehrmann and Vogel, 1998; Teixeira et al., 2013). The few carbohydrate transport enzymes that were characterized in heterofermentative lactobacilli include MFS permeases but no ABC-transporters or PTS systems (Chaillou et al., 1998; Djordjevic et al., 2001; Neubauer et al., 1994).

Homofermentative and heterofermentative lactobacilli co-exist in many of their natural and man-made habitats including sourdough, rice vinegar fermentations, and fermented vegetables (De Vuyst et al., 2014; Duar et al., 2017a; Wu et al., 2012; Zheng et al., 2015a). This co-existence has been described as complementary rather than competitive (Andreevskaya, 2017; Tannock et al., 2012) and may reflect resource partitioning by means of preferential utilization of different carbohydrates. However, only few studies describe mechanisms and regulation of carbohydrate transport and metabolism in heterofermentative lactobacilli as a prerequisite to understand the molecular basis of resource partitioning between homo- and hetero-fermentative lactobacilli (Gänzle and Follador, 2012). It was therefore the aim of this study to provide a genome-wide assessment of carbohydrate transport and metabolism in *Lactobacillus reuteri*, and to complement bioinformatic analyses by phenotypic characterization of carbohydrate utilization and quantification of gene expression. *L. reuteri* was used as model organism. The species *L. reuteri*

represents the *L. reuteri* group in the heterofermentative clade of lactobacilli (Duar et al., 2017b; Zheng et al., 2015a). Owing to its occurrence in the upper intestine of animals (Frese et al., 2011) and in fermented cereals (Su et al., 2012), *L. reuteri* is well characterized genetically and physiologically, and metabolic traits that contribute to its ecological fitness in cereal and intestinal ecosystems are well understood (Frese et al., 2011; Gänzle et al., 2007; Krumbeck et al., 2016; Lin et al., 2015; Lin and Gänzle, 2014). The study employed 7 strains of *L. reuteri* with known genome sequence (Zheng et al., 2015b).

3.2 Materials and Methods

3.2.1 Strains and growth conditions

The sourdough isolates *L. reuteri* LTH2584, LTH5448, TMW1.112 and TMW1.656, and rodent isolates *L. reuteri* 100-23, mlc3 and lpuph were routinely grown on mMRS medium. The sourdough isolates belong to the rodent-adapted lineages I and III (Zheng et al., 2015b); rodent isolates were selected from the same lineages. Working cultures were prepared by streaking cultures on mMRS agar from the -80 ºC glycerol stocks, followed by two subcultures in mMRS broth. The cultures were grown anaerobically at 37 ºC. The mMRS broth medium contained the following ingredients per liter: beef extract (5 g), yeast extract (5 g), peptone (10 g), malt extract (10 g), NH_4Cl (3 g), K_2HPO_4 (4 g), KH_2PO_4 (2.6 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $MnSO_4 \cdot 4H_2O$ (0.05 g), L-cysteine·HCl (0.5 g), Tween-80 (1 g), glucose (5 g), fructose (5 g) and maltose (10 g). All chemicals were obtained from Sigma Aldrich (Oakville, Ontario, Canada) unless otherwise specified. The pH was adjusted to 6.2 before autoclaving; solid media were prepared by adding 2 % agar.

Carbohydrate metabolism was analyzed in the Chemically Defined Medium (CDM) (Hüfner et al., 2008) that contained the following ingredients per liter: sodium acetate (6 g), KH_2PO_4 (3 g), K_2HPO_4 (3 g), $MgSO_4 \cdot 7H2O$ (0.2 g), $FeSO_4 \cdot 7H2O$ (0.01 g), Tween-80 (1 g), L-alanine (0.1 g), L-arginine (0.1 g), L-asparagine (0.2 g), L-asparic acid (0.2 g), L-cysteine (0.2 g), L-glutamine (0.2 g), L-glutamic acid (0.2 g), glycine (0.1 g), L-histidine (0.1 g), L-isoleucine (0.1 g), L-leucine (0.1 g), L-lysine (0.1 g), L-methionine (0.1 g), L-phenylalanine (0.1 g), L-proline (0.06 g), L-serine (0.1 g), L-threonine (0.1 g), L-tryptophan (0.1 g), L-tyrosine (0.1 g), L-valine (0.1 g), nicotinic acid (0.001 g), calcium pantothenate (0.001 g), pyridoxal (0.002 g), riboflavin (0.001 g), uracil (0.001 g), di-ammonium hydrogen citrate (2 g), NaCl (0.02 g), ascorbic acid (0.5 g), guanine (0.1 g), cytidine (0.1 g), 2'-deoxyadenosine (0.1 g), 2'-deoxyuridine (0.1 g), xanthine (0.1 g), inosine (0.1 g), $MnSO_4 \cdot H_2O$ (0.02 g), $CoCl_2$ (0.0046 g), cyanocobalamin (0.004 g), para-aminobenzoic acid (0.0006 g), myo-inositol (0.005 g), D-biotin (0.001 g), folic acid (0.0005 g), thymine (0.004 g), and sugars (at 2 g or 20 g). The pH of stock solutions was adjusted to 6.3 prior to sterilization. Of above compounds, sodium acetate, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and Tween-80 were prepared as stock solution which was autoclaved at 121 ^oC for 15 min. Stock solutions of sugars were sterilized with 0.22 μm filters; remaining ingredients were prepared as a concentrated stock solution, filter sterilized, and added to the final medium.

3.2.2 CAZyme annotation

Carbohydrate active enzymes in the genomes of 7 strains of *L. reuteri* were annotated in two platforms, the CAZymes Analysis Toolkit (http:/gg/mothra.ornl.gov/cgi-bin/cat.cgi) (Park et al., 2010) and the dbCAN prediction web server (http://csbl.bmb.uga.edu/dbCAN/) (Yin et al., 2012). Sequence-similarity-based method was used in both analysis platforms with default threshold of

e-value. Protein sequences were assigned to the respective CAZyme families if the annotation in the CAZymes Analysis and dbCAN prediction platforms was consistent.

3.2.3 Determination of carbohydrate utilization

Growth of *L. reuteri* in the CDM with different carbon sources was assayed by incubation in microtitre plates (Lin et al., 2015). In brief, overnight cultures were washed twice in saline and re-suspended to the same volume sterile saline; 20 µL of this cell suspension was inoculated into 96-well microtitre plates containing 180 µL of CDM with different carbon sources at 2 g/L. Plates were covered with 50 µL paraffin oil to exclude oxygen. Plates were incubated at 37 °C for 24 h in a microtitre plate photometer and the optical density at 600 nm was measured every 0.5 h.

The assay was performed in three biological replicates. The following carbon sources were evaluated: L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-lactulose, L-rhamnose, D-cellobiose, D-trehalose, D-gentiobiose, xylobiose, palatinose, D-raffinose, D-glucotriose, D-mannotriose, D-maltotriose, D-melezitose, isomalto-oligosaccharides (IMO), fructo-oligosaccharides (FOS), D-mannose, D-sorbitol, D-mannitol, inositol, glucuronic, amygdalin, dulcitol, D-gluconate, inulin, xylan, pectin, cellulose, starch, dextran, and galactan.

The carbohydrate fermentation profile was also assessed by using the API 50 CH system (bioMérieux, Inc., Marcy l'Etoile, France), according to the manufacturer's instructions. Enzyme profile was assayed with API ZYM system (bioMérieux, Inc., Marcy l'Etoile, France).

3.2.4 Bioinformatics analysis of sugar transporters

Genomes of the 7 strains of *L. reuteri* were downloaded from NCBI FTP website (ftp://ftp.ncbi.nlm.nih.gov/genomes/), and built as local databases using standalone BLAST+,

2.2.30 (Camacho et al., 2009). Sequences of prokaryotic transporter enzymes were retrieved from the Transporter Classification Database (TCDB, http://www.tcdb.org/) (Saier et al., 2014). TCDB transporter sequences were used as query sequences for blastp search of the *L. reuteri* genomes with standalone BLAST+, 2.2.30 with a cutoff of e^{-10}. Initial hits were used as query sequences for blastp search of the TCDB database for further confirmation or exclusion. Genome-wide annotation of membrane transporters, including sugar transporters and non-sugar transporters, was performed using Transporter Automatic Annotation Pipeline, TransAAP in Transporter Database (Ren et al., 2007). The predicted, substrate-specific, sugar transports above would be further verified if their corresponding properties are consistent with the annotation or supporting evidence of TransAAP. Characterized proteins with homologies to the predicted sugar transporters were retrieved from Uniprot database (http://www.uniprot.org/blast/) to support predictions of substrate specificity.

3.2.5 Quantification of gene expression during growth of *L. reuteri* in sourdough

To determine which sugar transporters are expressed during growth in sourdough, mRNA was quantified by reverse transcription-quantitative PCR (RT-qPCR). Whole wheat sourdough was prepared by mixing 10 g of whole wheat flour with 10 mL of a cell suspension of *L. reuteri* LTH5448, 100-23, or TMW1.656 in tap water to achieve an initial cell count of about 10^7 CFU/g (Lin et al., 2015). Dough fermented with *L. reuteri* LTH5448 was fermented with and without addition of 2 % baker's yeast. The dough was fermented at 37°C until the pH was reduced to 4.5, corresponding to the exponential phase of growth. Cells were isolated from sourdoughs as described (Teixeira et al., 2014) and RNA was extracted using RNAprotect Bacteria Reagent and RNeasy Minikit (Qiagen, USA) prior to DNAase treatment with RQ1 RNase-Free DNase Kit (Promega, Madison, USA) to eliminate residual DNA. RNA quality and quantity were assessed

spectroscopically (Nanodrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to reverse transcription to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, USA). Quantitative PCR was performed with the QuantiFast SYBR Green master mix (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Burlington ON) with primers and annealing temperatures shown in Table 3-1 below. DNase-treated RNA samples served as negative controls. Relative gene expression was calculated as

$$\frac{(E_{target})^{\Delta CT(reference-sample)}}{(E_{reference})^{\Delta CT(reference-sample)}}$$

Where E_{target} is the PCR efficiency for the target gene, $E_{reference}$ is the PCR efficiency for the housekeeping gene, and ΔCT is the threshold cycle for samples obtained at sample and reference conditions (Pfaffl, 2001). Exponentially growing cultures (OD_{600nm} 0.4-0.6) in CDM-glucose (10 g/L) were used as reference conditions and *pho* coding for phosphoketolase was used as the housekeeping gene. The experiment was performed in triplicate independent experiments, each analyzed in duplicate PCR reactions.

Table 3-1 Strain characteristics, genome features, and carbohydrate active enzymes (CAZymes) of strains of *L. reuteri*

	LTH2584	**LTH5448**	**TMW1.656**	**TMW1.112**	**100-23**	**mlc3**	**lpuph**
Source	Sourdough	Sourdough	Sourdough	Sourdough	Rodent intestine	Rodent intestine	Rodent intestine
Lineage[a]	III	I	III	III	III	III	I
Genome size	2.07 Mb	1.90 Mb	1.94 Mb	2.03 Mb	2.31 Mb	2.02 Mb	2.12 Mb
Protein number	1803	1699	1632	1745	2049	1805	1918
GH[b]	13	12	13	13	16	15	14
GT[b]	6	12	9	9	13	12	12
Esterase	1	1	1	2	1	0	1
Carbohydrate-binding module	2	2	2	3	3	3	2
CAZyme number (% total protein)	22 (1.2%)	27 (1.6%)	25 (1.5%)	27 (1.5%)	33 (1.6%)	30 (1.6%)	29 (1.5%)
GH families (number proteins in one family)	GH2(2), GH8, GH13(2), GH31, GH36, GH42, GH65	GH2, GH8, GH13(2), GH31, GH36, GH42, GH65, GH68	GH2, GH8, GH13(2), GH31, GH36, GH42, GH65, GH68, GH70	GH2, GH8, GH13(2), GH31, GH36, GH42, GH53, GH65, GH68, GH70	GH2(2), GH8, GH13(2), GH31, GH36, GH42, GH65, GH68, GH70	GH2(2), GH8, GH13(2), GH31, GH36, GH42, GH65, GH68, GH70	GH2(2), GH13(2), GH31, GH36, GH42, GH53, GH65, GH68, GH70
Genome accession# in NCBI	NZ_JOSX01000020.1	NZ_JOOG01000004.1	NZ_JOSW02000004.1	NZ_JOKX02000004.1	NZ_AAPZ02000001.1	NZ_AEAW01000043.1	NZ_AEAX01000045.1

[a] Lineages were assigned based on core-genome alignment (Zheng et al., 2015b).
[b]GH, glycosyl hydrolase; GT, glycosyl transferase. None of the genomes contained genes annotated as members of the polysaccharide lyase (PL) family or the auxiliary activity (AA) family.

3.2.6 Effect of the carbohydrate source on expression of sugar transporters

To determine the effect of the carbohydrate source on gene expression in *L. reuteri* 100-23 and LTH5448, strains were grown in CDM broth containing 20 $g \cdot L^{-1}$ raffinose, maltose, sucrose, melibiose, or lactose, or 10 $g \cdot L^{-1}$ maltose and 10 $g \cdot L^{-1}$ xylose. Cultures were grown to an OD600nm of 0.4; cells were harvested for isolation of RNA and quantification of mRNA by RT-qPCR as described above. Results were indicated as means ± standard deviations for 5 technical replicates from two biological replicates.

3.2.7 Global reconstruction of metabolism pathway

Carbohydrates metabolism pathways were visualized through KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/kaas-bin/kaas_main) (Moriya et al., 2007) and manually curated to match metabolic pathways in heterofermentative lactobacilli (Zheng et al., 2015a). For KAAS analysis, proteins encoded by the genomes were searched against defined gene data sets using bi-directional best hit (BBH)-based GHOSTX program.

3.2.8 Multiple alignment for genomes or protein domains

Multiple alignment of genomes was performed using standalone BLAST+, 2.2.30. Genome sequences were concatenated with Geneious (6.1.6) prior to alignment. Target protein domains (Finn et al., 2014) of glycosyl hydrolase family 53 proteins and glycosyl hydrolase family 8 proteins were aligned with in MEGA 6.

3.2.9 Statistical analysis

The one-way ANOVA (SigmaPlot, version 12.5) was used for statistical analysis. Statistical analysis was performed using Student's t-test (SigmaPlot, version 12.5) and significant differences in gene expression were evaluated with a 5 % probability of error ($P < 0.05$).

3.3 Results

3.3.1 Genomic and phenotypic characteristics of carbohydrate metabolism of *L. reuteri*

Genome scale annotation of carbohydrate active enzymes (CAZymes) was conducted for sourdough- and rodent-isolates of *L. reuteri* (Table 3-1 above). The genome size or the number of protein coding sequences was not significantly different between strains of different lineages; the number of carbohydrate active enzymes relative to the total number of predicted proteins ranged from 1.2 to 1.6 %. CAZymes in *L. reuteri* consisted mainly of glycoside hydrolases (GH) and glycosyl transferases (GT). Only few carbohydrate esterases (CE) and carbohydrate-binding modules (CBM) were present; polysaccharide lyases and auxiliary activity family proteins were absent (Table 3-1 above).

The presence of glycosyl hydrolases largely overlapped between the 7 strains of *L. reuteri* (Table 3-1 above). Strain-specific differences related to levansucrases and reuteransucrases in the GH68 and GH70 families, and a predicted GH53 family endo-β (1→4)-galactanase that was present in *L. reuteri* TMW1.112 and lpuph (Table 3-1 above). A CBM50 family protein which attaches cell-wall associated hydrolases to peptidoglycan was present in all strains; a CBM37 family protein

was identified only in the genome of *L. reuteri* 100-23. This protein was previously considered to be unique to the cellulose-degrading *Ruminococcus albus* (Ezer et al., 2008). The phenotype of carbohydrate utilization in the 7 strains of *L. reuteri* was evaluated by API 50CH and growth in chemically defined medium (Table 3-2 below, and Table 3-5 below). Not all of the carbohydrates that were fermented in the API 50CH assay supported growth in chemically defined medium probably due to limited nutrition of the chemically defined medium.

The capacity to metabolize arabinose and xylose differed between strains of *L. reuteri*. Comparison of the genotype of xylose and arabinose-utilizing strains with the genotype of *L. reuteri* LTH2584 and LTH5448, which were unable to ferment arabinose and xylose, respectively, demonstrated that the lack of pentose utilization results from the loss of operons coding for enzymes involved in pentose utilization (Figure 3-1 below).

L. reuteri is capable of growing on isomalto-oligosaccharides (IMO) with a degree of polymerization up to 6 (Hu et al., 2013) and raffinose (Teixeira et al., 2012), but unable to utilize polysaccharides. In keeping with the genotype and the fermentation patterns, the API ZYM assay identified esterase, α-glucosidase, α-galactosidase and β-galactosidase activity (Table 3-6 below).

Table 3-2 Carbohydrate utilization profile for cereal and rodent strains of *L. reuteri*

Sugars	LTH2584		TMW1.112		TMW1.656		LTH5448		100-23		lpuph		mlc3	
	API	Growth	API	Growth	API	Growth	API	Growth	API	Growth	API	Growth	API	Growth
L-arabinose	-	-	+	-	-	-	+	+	+	+	+		+	
D-ribose	+	n/d	+	n/d	+	n/d	+	n/d	+	n/d	+		+	
D-xylose	+	-	-	-	-	-	-	-	+	-	-		+	
D-galactose	+	+	+	+	+	+	+	+	+	+	+		+	
D-glucose	+	+	+	+	+	+	+	+	+	+	+		+	
Methyl-αD-Glucopyranoside	+	n/d	-	n/d	-	n/d	-	n/d	-	n/d	-		-	
D-maltose	+	+	+	+	+	+	+	+	+	+	+	n/d	+	n/d
D-lactose	+	+	+	+	+	+	+	+	+	+	+		+	
D-melibiose	+	+	+	+	+	+	+	+	+	+	+		+	
D-sucrose	+	-	+	+	+	+	+	+	+	+	+		+	
D-raffinose	+	+	+	+	+	+	+	+	+	+	+		+	
Potassium gluconate	+	-	+	-	+	-	+	+	+	-	-		+	
Isomalto-oligosaccharides (IMO)	n/d	+	n/d	+	n/d	+	n/d	+	n/d	+	n/d		n/d	
D-lactulose	n/d	-	n/d	-	n/d	+	n/d	+	n/d	+	n/d		n/d	
Palatinose hydrate	n/d	+	n/d	+	n/d	+	n/d	+	n/d	+	n/d		n/d	

Carbohydrates with negative result for all strains in the API 50CH assay: glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-Mannopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, ferric citrate, salicin, D-cellobiose, D-trehalose, inulin, D-melezitose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, potassium 5-ketogluconate. **Carbohydrates with negative result for all strains in the growth test**: D-xylose, L-rhamnose, D-cellobiose, D-trehalose, D-maltotriose, D-fructose, D-mannose, D-sorbitol, D-mannitol, inositol, gentiobiose, D-melezitose monohydrate, glucuronic, amygdalin, dulcitol, inulin, xylan, pectin, cellulose, starch, dextran, glucotriose, xylobiose, mannotriose, galactan and fructo-oligosaccharides (FOS). +: growth; -: no growth; **n/d**: not determined in this assay.

Table 3-3 *In silico* identification of putative sugar transporters in strains of *L. reuteri*

Substrates	Protein symbols[b]	# of TMSs in queries	Query length (aa)	Accession # (UniProt)	Identity (%)	Transp. families[c]	Locus tag[d]						
							LTH2584	TMW1.112	TMW1.1656	LTH5448	100-23	mlc3	lpuph
D-ribose	**RbsD2**	11	452	Q8XEV7	51.5	FHS	**LR3_02825**	HF82_05735	HQ33_08370	HN00_00840	Lreu23DRAFT_3462	cds1089	2506440742
L-arabinose	AraE1	12	387	P31122	29.8	DHA1	**LR3_05370**	HF82_07440	HQ33_00710	HN00_07160	Lreu23DRAFT_4690	cds545	2506439633
	AraE	12	472	C4B4V9	72.7	SP	-	HF82_06075	-	HN00_01255	Lreu23DRAFT_3536	cds38	2506439466
	AraE2	12	435	P96710	30.6	SP	**LR3_04700**	HF82_04025	HQ33_00215	HN00_04590	Lreu23DRAFT_4584	cds334	2506439802
D-xylose	**XylT**	12	466	O52733	67.2	SP	**LR3_02915**	-	-	-	Lreu23DRAFT_3480	cds874	-
	XynT	11	500	P96792	35.3	GPH	**LR3_02925**	-	-	-	Lreu23DRAFT_3482	cds877	-
D-glucose	**GlcU**	10	288	P40420	38.0	GRP	**LR3_07890**	HF82_06485	HQ33_06895	HN00_06430	Lreu23DRAFT_4885	-	2506440027
	GlcU1	10	287	A0A0E0ZTM0	38.6	GRP	**LR3_02885**	HF82_05795	HQ33_08220	HN00_00900	Lreu23DRAFT_3474	cds1077	2506440754
	GlcU2	12	392	Q04DP6	26.8	GT	**LR3_07180**	HF82_08080	HQ33_03965	HN00_02370	Lreu23DRAFT_4316	-	(2506440383… 2506440382)
D-galactose	**GalP**	13	651	Q9X761	41.8	GPH	**LR3_04320**	HF82_07560	HQ33_00580	n.a.[e]	Lreu23DRAFT_4663	cds570	2506440314
D-maltose	**MalT**	12	450	Q9A612	44.9	GPH	**LR3_08075**	HF82_06335	-	HN00_04890	Lreu23DRAFT_4916	cds783	2506440002
MOS[a]	**MalT1**	12	462	Q9A612	27.2	GPH	**LR3_09920**	HF82_08930	HQ33_08125	HN00_03255	Lreu23DRAFT_4937	cds1065	2506440913
	MalT2	12	456	Q8EEC4	27.6	GPH	**LR3_09935**	HF82_08945	HQ33_08120	HN00_03240	Lreu23DRAFT_4938	cds1067	2506440910
D-sucrose	**ScrT**	12	406	Q04DP6	26.8	GT	**LR3_01255**	HF82_04270	HQ33_08175	HN00_01910	Lreu23DRAFT_4471	cds1267	2506440958
D-lactose/ D-raffinose	**LacS**	12	641	P23936	38.2	GPH	**LR3_00360**	HF82_02760	HQ33_03895	HN00_02605	Lreu23DRAFT_5165	cds1091	2506439790
D-melibiose	SotB1	12	387	Q9S3J9	29.6	DHA1	**LR3_04870**	HF82_10260	HQ33_00040	HN00_03045	Lreu23DRAFT_4547	cds368	2506440515
	SotB2	12	390	Q9S3J9	26.2	DHA1	**LR3_04495**	HF82_04330	HQ33_00405	HN00_07465	Lreu23DRAFT_4624	cds663	2506440701
D-gluconate	**GntP**	9	379	P12012	55.1	GntP	**LR3_09825**	HF82_00365	HQ33_07405	HN00_05280	Lreu23DRAFT_3217	cds1449	2506440538

[a] MOS: maltooligosaccharides; [b] Gene symbols are printed in in bold if they are located adjacent to functionally related genes; other genes are not located in proximity to functionally related gene (clusters); [c] FHS: Fucose: H^+ Symporter Family; DHA1: drug: H^+ antiporter-1 Family; SP: Sugar Porter Family; GPH: Glycoside-Pentoside-Hexuronide: Cation Symporter Family; GT: Glucose Transporter Family; GntP: Gluconate:H+ Symporter (GntP) Family; [d] The genes that were used as queries for Blastp anlaysis are printed in bold; "-"indicates absence of genes; genes in different strains with identical symbols share homology of 98% or greater; [e] GalP is present in LTH5448 but not annotated in genome file uploaded to Genbank.

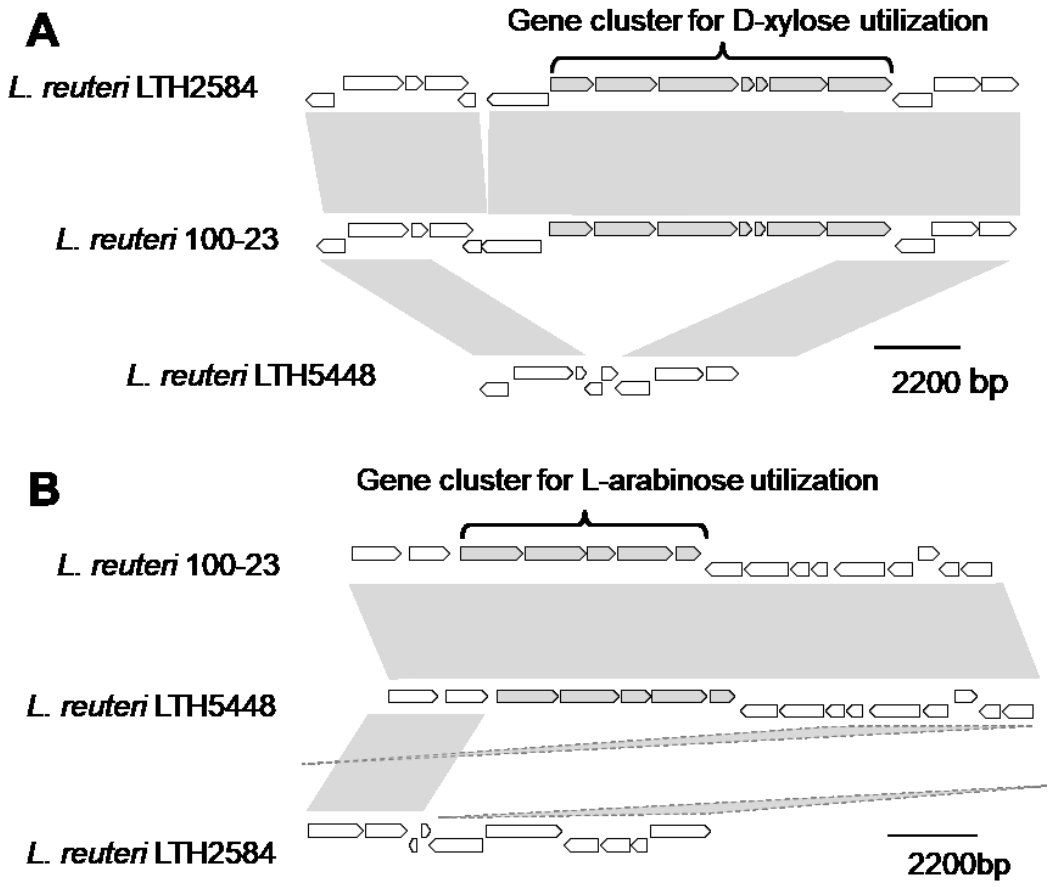

Figure 3-1 Comparison of the pentose utilization gene cluster and surrounding genomic regions in pentose fermenting and pentose negative strains

Panel A: Cluster for xylose utilization *L. reuteri* LTH2584, 100-23, and LTH5448. **Panel B**: Cluster for arabinose utilization in *L. reuteri* 100-23, LTH5448, and LTH2584. Gray areas connect genomic regions with high (>98%) nucleotide identity. Gene names are provided in Figure 3-5 below.

3.3.2 *In silico* analysis of sugar transporters

Transport enzymes for carbohydrates were initially predicted *in silico* (Table 3-3 above). Transporters predominantly belonged to the major facilitator superfamily while few transporters belonged to the drug/metabolite transporter (DMT) superfamily (Table 3-3 above). Members of other transporter families, particularly PTS and ABC-type transporters, were absent. The substrate

for the transporters was inferred from the protein homology to experimentally characterized transport enzymes, and by analyzing whether the genes are part of a sugar utilization operon (Table 3-3 above and Figure 3-5 below). The strain-specific absence of genes coding for L-arabinose and D-xylose transporters corresponded to the inability of the strains to ferment the corresponding sugars (Figure 3-1 above, Table 3-2 above, Table 3-3 above, and Table 3-5 below).

Table 3-4 Sequence of primers used to quantify mRNA levels of putative sugar transporters

Gene		Primers (5'-3')	Annealing temp. (^{o}C)	Amplicon size (bp)	PCR efficiency
rbsD2	rbsD2_F/R	AACAATTCAAGGACGGGTATCA / GGTGCTCAGTCCAGAAGTAAAT	62	107	2.04
araE1	araE1_F/R	CACTTGGCTGGCTCCTATTT / CCGCTTGTCCATTGGTGTAA	62	107	2.08
araE	araE_F/R	GCTTCTCTCGTTGGTTGGATTA / TTCCGCCGACCAAACTTATC	62	98	2.00
araE2	araE2_F/R	CCAGTGGAGTTGGTACTTGTAT / GACCGCCAAGATGAGTTAAGA	62	90	1.97
xylT	xylT_F/R	GTCTCATTAACATTCCCTCCTCTAC / TGGAGTGGACGAACCAAATAG	60	103	2.01
xynC	xynC_F/R	GTCTTTCTTTGGCCGCTTATTC / AAATGCTGGGAAAGACCAAATC	60	117	2.24
glcU	glcU_F/R	CCGACAAACGACGTCATAACTA / TTGGACAGGTTGGTCAGTTC	62	100	2.05
glcU1	glcU1_F/R	GATCAGAGCAGCGGAAAGAA / CATCTTTGGGAATGCTGAGTAAAC	62	96	2.00
glcU2	glcU2_F/R	GCACGCTGTCTTTCTTGTTTAT / TCACTGGTTGGACGGATTTAG	62	143	2.04
galP	galP_F/R	CTTCTCTACTCGTCACGCAATC / ATCCAGTACCACGAAGCTTAAC	62	98	2.07
malT	mal_F/R	CCTTGGCTGGTTCTTCATCT / GCCCATGTACGGTCTGAATAA	60	84	1.93
malT1	malT1_F/R	GCAGTGAGAAAGCCATGTTTATT / CGAAGCAGGTTGATCTGGATAC	62	102	1.99
malT2	malT2_F/R	CTGAGAACTCTGCAGTGAGAAA / GGTTGATCTGGATACAGGGATG	62	106	1.91
scrT	suc_F/R	TTGCCTTCCTCTTGGTTGTAG / CAGTATAGCTGCTGCCCTTAAT	60	87	1.98
lacS	lacY_F/R	GGGTTGATTACTGGGTTGATTG / CCACCGGGTCTTCGTATTATC	60	96	2.10
sotB1	sotB1_F/R	GCTGATCGGGAATATCCAGAAG / TACTGATCGATGCCGTCAAAG	60	103	1.95
sotB2	sotB2_F/R	CCGTTATCAGCACTACCCTTAC / TGGGACGAGCCAAATCAAG	60	95	2.11
gntP	gntP_F/R	CGCTAACCTTGGACACGTATTA / ACGGTAAACACGCGGATAAA	62	115	1.99
phok	phk_F/R	GTCCAGACCTCGTTAAGGAATAC / CGTGGGTGCTTAGAAGTTACA	60	118	2.04

3.3.3 Analysis of the expression of genes coding for sugar transporters in sourdough and during growth on defined carbohydrate sources

Quantification of the expression of predicted transporter genes aimed to determine whether particular transporters are over-expressed in response to the respective substrates. Expression was initially quantified in *L. reuteri* growing in sourdough, a carbohydrate-rich ecosystem which represents the origin of the strains or resembles the rodent forestomach with respect to the carbohydrate availability (Schwab et al., 2014; Tannock et al., 2012). Two sourdough isolates and one rodent isolate were selected for analysis of gene expression; gene expression of *L. reuteri* LTH5448 was additionally analyzed after addition of baker's yeast. All 17 genes were expressed in at least one of the strains (Figure 3-2 below); the addition of baker's yeast, which rapidly depletes glucose during growth in sourdough and thus alleviates carbon catabolite repression, did not alter expression of genes coding for carbohydrate transporters. Relative to growth with glucose as sole carbon source, genes coding for transporters ScrT, LacS, MalT, MalT1 and MalT2, which code for transporters specific for sucrose, raffinose and lactose, and maltose or malto-oligosaccharides (Table 3-3 above), were significantly overexpressed during growth of one or several strains in sourdough (Figure 3-2 below). Conversely, the expression of genes coding for enzymes transporting glucose, gluconate and ribose was significantly reduced in sourdough (Figure 3-2 below).

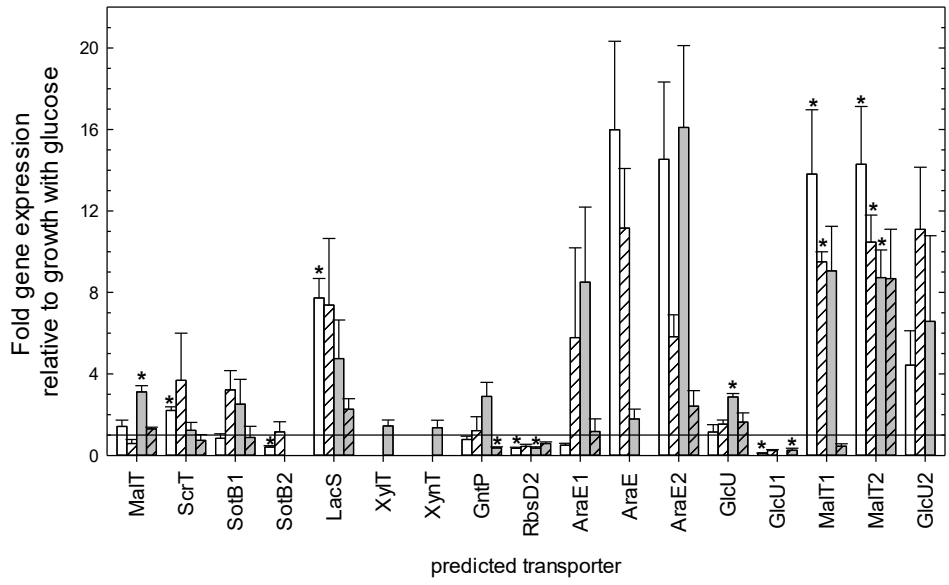

Figure 3-2 Expression of predicted transporter genes by *L. reuteri* during growth in sourdough

White bars, *L. reuteri* LTH5448, **white, hatched bars**, *L. reuteri* LTH5448 with baker's yeast, **gray bars**, *L. reuteri* 100-23, **gray, hatched bars**, *L. reuteri* TMW1.656. Sourdoughs were fermented at 37°C until the dough pH reached pH 4.5, corresponding to the exponential phase of growth. Relative gene expression was quantified relative to the expression in chemically defined medium with glucose as sole carbon source; the horizontal line represents unity (gene expression equivalent at the reference conditions). Results are shown as means ± standard error of triplicate biological repeats, each sample was analyzed in technical duplicates. Genes that were differentially expressed ($P < 0.05$) relative to expression by the same strain at reference conditions are marked with an asterisk. Genes coding for XylT and XynT are present only in *L. reuteri* 100-23.

Gene expression of 7 genes was analyzed during growth of two strains in chemically defined medium with different carbohydrate sources (Figure 3-3 below). Transport genes and substrates were selected to include genes with uncertain substrate assignment, or genes that were highly expressed during growth in sourdough. Overexpression of *malT* was only observed in presence of maltose but not in response to other sugars. Sucrose and raffinose induced expression of *scrT*. Induction by raffinose corresponds to intracellular sucrose release when raffinose is metabolized intracellularly by α-galactosidases (Teixeira et al., 2012), however, in *L. reuteri* LTH5448, *scrT*

expression was also induced by melibiose (Figure 3-3 below). The gene coding for the lactose transporter *lacS* was induced by lactose in both strains but also by raffinose and melibiose in *L. reuteri* 100-23, suggesting a broad substrate specificity of this transporter (Figure 3-3 below). Expression of *sotB1*, *sotB2*, *xylT* and *xynT* was below the detection limit, or did not respond to the predicted substrates. Overall, the quantification of gene expression conforms to the assignment of *malT*, *scrT* and *lacS* as transporters for D-maltose, D-sucrose and D-lactose, respectively. D-raffinose and melibiose are likely additional substrates of *lacS*.

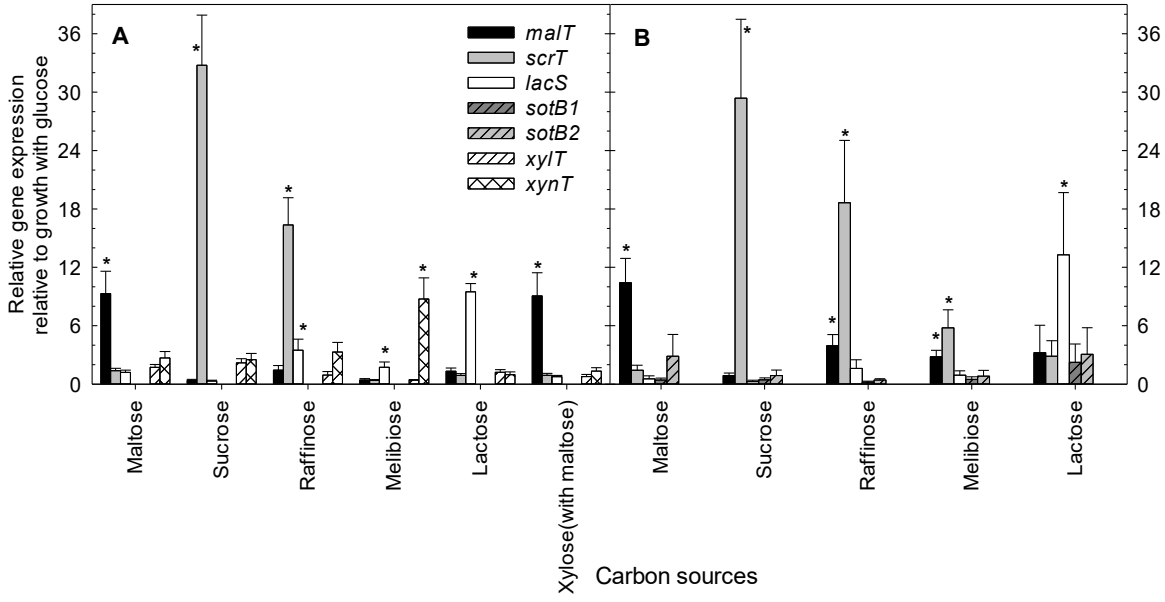

Figure 3-3 Gene expression of predicted sugar transporters of *L. reuteri* in the chemically defined medium

Gene expression was quantified in *L. reuteri* 100-23 (**Panel A**) and LTH5448 (**Panel B**) growing in chemically defined media with different carbon sources relative to expression in medium with glucose. Data represent mean ± SD of 5 replicates. Genes that were differentially expressed ($P < 0.05$) relative to expression by the same strain at reference conditions are marked with an asterisk. The mRNA levels of *sotB1* or *sotB2* in samples of *L. reuteri* 100-23 were below the detection limit.

3.3.4 Protein sequence analysis for two novel glycoside hydrolases involved in oligosaccharide utilization

The CAZyme annotation identified two novel glycoside hydrolases which are not characterized biochemically in lactic acid bacteria: a licheninase (EC 3.2.1.73) and an endo-(1→4)-β-galactosidase (EC 3.2.1.89) (Table 3-1 above). The functions were predicted by identifying conserved functional domains as well as signature patterns in the deduced protein sequences (data not shown) and by alignment of the proteins with homologous proteins (Figure 3-6 below). The putative licheninase in *L. reuteri* TMW1.112 is a GH53 family protein with 419 amino acids. A phylogenetical tree based on multiple protein alignments (Figure 3-6 below) revealed that the proteins are homologous to licheninases and endo-β-galactosidases in bacilli and clostridia, respectively, and more distantly related to enzymes in Gram-negative bacteria and fungi (Figure 3-6 above). The major signature patterns as well as key active sites were conserved in the catalytic domains of the predicted enzyme (data not shown). *In silico* analysis of the cellular location indicated that the licheninase includes a transmembrane helix and may be membrane bound. The protein sequence of the endo-β-galactosidase includes a signal peptide and is thus likely an extracellular enzyme.

3.3.5 Global pathway for carbohydrate utilization in *L. reuteri*

Genomic data, information on the effect of substrate on gene expression, and phenotypic and genomic data provided in this and previous studies (Table 3-3 above, Table 3-4 above, Figure 3-3

above, Table 3-5 below, and Table 3-6 below) (Cardelle-Cobas et al., 2011; Gänzle, 2015; Hu et al., 2013; Teixeira et al., 2012; Zheng et al., 2015a) was used to depict carbohydrate transport and metabolism (Figure 3-4 below). Pending characterization of the putative membrane bound or extracellular licheninase and endo-β-galactosidase, fructansucrases and reuteransucrases are the only extracellular glycosyl hydrolases (Figure 3-4 below). Fructansucrases use sucrose or raffinose-family oligosaccharides as substrate (Teixeira et al., 2012) while glucansucrases including reuteransucrase use only sucrose. Oligosaccharides, the preferred substrate for growth of *L. reuteri* (Gänzle et al., 2007), are transported by ScrT, LacS, MalT, MalT1 and MalT2. Analysis of gene expression provided here and elsewhere suggests that melibiose and raffinose-family oligosaccharides are transported by LacS and/ or ScrT (Figure 3-3 above and Figure 3-4 below). The putative transporters SotB1 and SotB2 have only low homology to biochemically characterized enzymes, are not highly expressed, and their expression is not altered in response to carbohydrates present in the substrate (Table 3-3 above, Figure 3-2 above, and Figure 3-3 above).

Genomes of *L. reuteri* code for sucrose- and maltose-phosphorylase, intracellular α-glucosidase and intracellular α- and β-galactosidases (Figure 3-4 below). Other intracellular glycosyl hydrolases are absent, in keeping with the fermentation pattern of the strains. The phosphoketolase pathway is the only pathway for conversion of hexoses and pentoses; galactose and pentoses are shunted into the phosphoketolase pathway with substrate-specific enzymes (Figure 3-4 below).

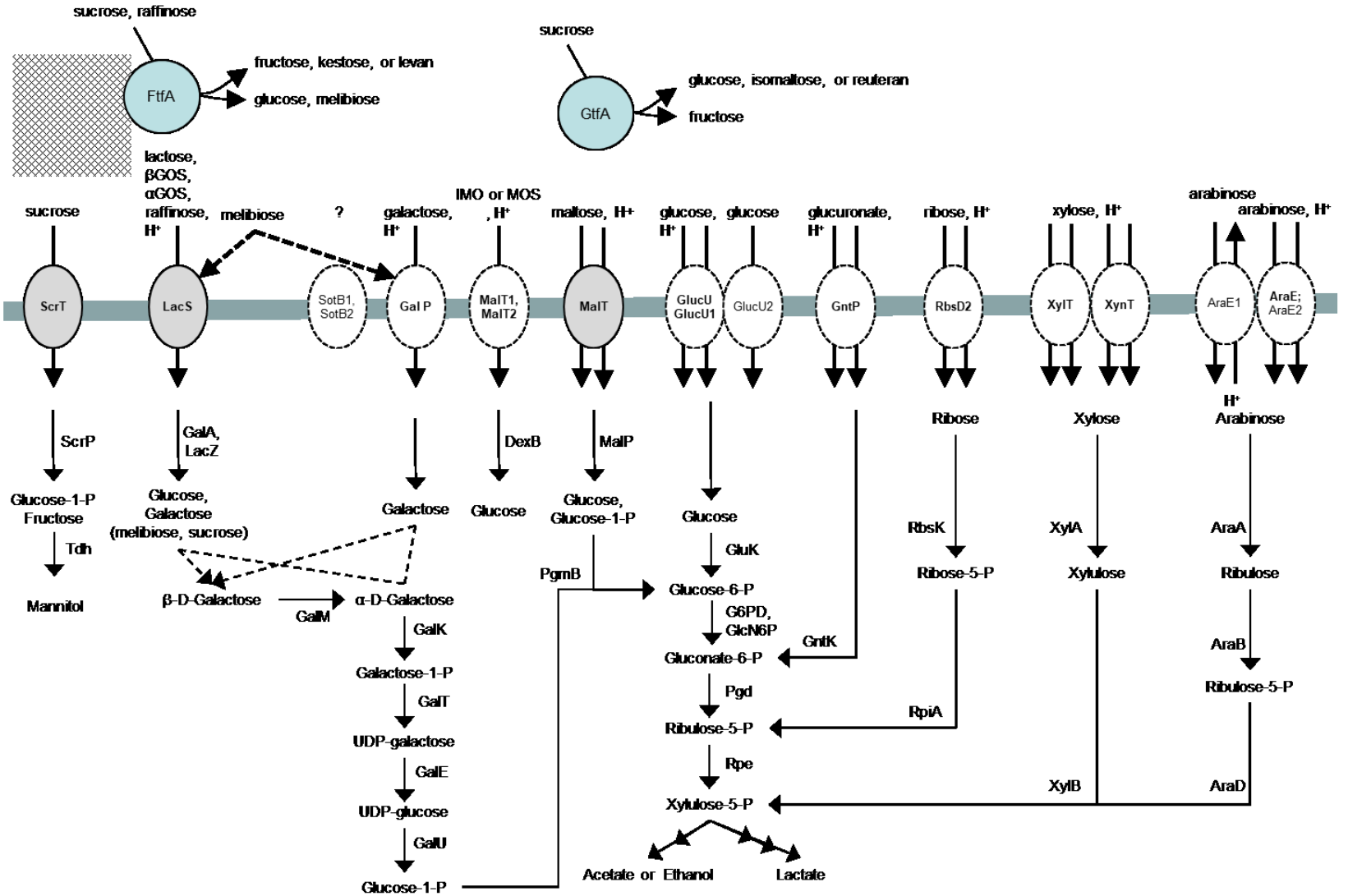

Figure 3-4 Overview of carbohydrate transport and metabolism in *L. reuteri*

Putative transport proteins which are located in an operon specific for the predicted substrate are printed in bold. Putative transport proteins which were overexpressed during growth on the predicted substrate are highlighted in grey. Note that several transport proteins have no known function or are redundant while at least two substrates, melibiose and fructose, could not be assigned to transport proteins. Enzymes and transporters are annotated as follows:

Sugar transporters: **ScrT**: D-sucrase transporter; **LacS**: D-lactose/ D-raffinose transporter; **SotB1**/**SotB2**: putative D-melibiose transporter; **GalP**: D-galactose transporter; **MalT**: D-maltose transporter; **MalT1**/**MalT2**: putative (iso)-malto-oligosaccharides transporter; **GlucU**/**GlucU1**/**GlucU2**: D-glucose transporters; **GntP**: D-gluconate transporter; **RbsD2**: D-ribose transporter; **XylT/XynT**: D-xylose transporter; **AraE1**/**AraE**/**AraE2**: (putative) L-arabinose transporters.

Sucrose/fructose metabolism: ScrP: sucrose phosphorylase; **Tdh**: mannitol dehydrogenase; **FtfA**: levansucrase, **GtfA**: reuteransucrase.

Galactose/lactose/GOS/raffinose metabolism: **GalA**: α-galactosidase; **LacZ**: β-galactosidase; **GalM**: aldose 1-epimerase; **GalK**: galactokinase; **GalT**: galactose-1-phosphate uridylyltransferase; **GalE**: UDP-glucose 4-epimerase; **GalU**: UDP-glucose pyrophosphorylase

Glucose/gluconate/maltose/IMO metabolism and phosphoketolase pathway: DexB: α (1→6)-glucosidase; **MalP**: maltose phosphorylase; **PgmB**: β-phosphoglucomutase; **GluK**: glucokinase; **G6PD**: glucose-6-phosphate 1-dehydrogenase; **GlcN6P**: 6-phosphogluconolactonase; **Pgd**: 6-phosphogluconate dehydrogenase; **Rpe**: ribulose-phosphate 3-epimerase; **GlnK**: gluconokinase.

Pentose metabolism: **Rbsk**: ribokinase; **RpiA**: ribose 5-phosphate isomerase A; **XylA**: xylose isomerase; **XylB**: xylulose kinase; **AraA**: L-arabinose isomerase; **AraB**: ribulokinase; **AraD**: L-ribulose-5-phosphate 4-epimerase.

3.4 Discussion

This study matched carbohydrate fermentation in *L. reuteri* with a genome-wide analysis of carbohydrate active enzymes and carbohydrate transporters and the quantification of genes coding for transport enzymes during growth in sourdough. This analysis revealed that carbohydrate transport in the heterofermentative *L. reuteri* differs substantially from the homofermentative model organisms *L. plantarum* and *L. casei*, and thus contributes to the molecular understanding of co-existence and resource partitioning of homofermentative and heterofermentative lactobacilli in food fermentations.

3.4.1 Genotypes of *L. reuteri* match phenotype of carbohydrate utilization

The sugar fermentation profile of *L. reuteri* strains matches the genotype. The lack of extracellular polysaccharide degrading enzymes reflects the adaptation *L. reuteri* to nutrient rich segments of the upper intestine of animals (Duar et al., 2017a; Frese et al., 2011; Walter, 2008), which contains high concentrations of fermentable carbohydrates including maltose, sucrose, and raffinose family oligosaccharides (Schwab et al., 2014; Tannock et al., 2012). These oligosaccharides are also the main carbohydrate sources in wheat and rye sourdoughs (Gänzle, 2014). The success of host-adapted *L. reuteri* in cereal fermentations (Su et al., 2012; Zheng et al., 2015b) is thus also explained by substrate profiles that match intestinal ecosystems, and the highly efficient metabolism of maltose, sucrose and raffinose (Gänzle et al., 2007). Strains of *L. reuteri* match to host adapted lineages (Duar et al., 2017a); maiantenance of lactose metabolism in *L. reuteri* likely

reflects the availability of lactose in the intestine of neonate mammals, contrasting the adaptation of *L. delbrueckii* subsp. *bulgaricus* to lactose-rich dairy environments (Van de Guchte et al., 2006). Strain specific differences were observed for pentose utilization; phenotypic observations were confirmed by matching deletions of the corresponding pentose utilizing genes. The carbohydrate fermentation patterns did not differentiate strains of *L. reuteri* based on their origin or lineage. Likewise, sourdough and intestinal isolates were not differentiated based on their carbohydrate metabolism (Zheng et al., 2015b) and carbohydrate catabolic enzymes were not identified among genes that are specific to host adapted lineages of *L. reuteri* (Frese et al., 2011). Propanediol and glycerol metabolism, however, is specific to human and chicken lineage strains of *L. reuteri*. In humans, *L. reuteri* are found in the carbohydrate-restricted lower intestine (Flint et al., 2008; Walter, 2008). In the human colon, propanediol is available as bacterial metabolite of fucose or rhamnose and supports trophic relationships between propanediol producing and propanediol utilizing bacteria (Schwab et al., 2017). Extracellular or cell wall-associated polysaccharide hydrolases in lactobacilli are limited to the exceptional and strain-specific occurrence of amylases or β-fructosidases (Gänzle and Follador, 2012; Goh et al., 2007); extracellular pentosanases or β-glucosidases have not been characterized biochemically in lactobacilli. Extracellular fructansucrases contribute to metabolism of raffinose-family oligosaccharides but not to polysaccharide degradation (Teixeira et al., 2012). Two strains of *L. reuteri* harbored genes coding for an extracellular licheninase and an endo-(1 → 4)- β -

galactosidase. The biochemical characterization of these enzymes and their implications for ecological fitness, however, remain subject to future investigations.

3.4.2 Identification of enzymes for carbohydrate transport

Sugar transport in bacteria is catalyzed by ABC transporters, secondary carriers and PTS (Saier, 2000). All sugar transporters identified in *L. reuteri* were secondary carriers and belonged predominantly to the major facilitator superfamily (MFS) (Pao et al., 1998). Members of the MFS use the proton motive force as energy source for transport (Konings, 2002; Pao et al., 1998). A striking feature of the carbohydrate metabolism in *L. reuteri* is the complete absence of ABC transporters or PTS systems (Table 3-3 above), which are the mainstay of carbohydrate transport in homofermentative lactobacilli (Andersson et al., 2005; Monedero et al., 2008). The preferential use of secondary carriers over PTS systems is shared by other heterofermentative lactobacilli (Zheng et al., 2015a) and the heterofermentative *Oenococcus* and *Leuconostoc* (Kim et al., 2011; Zaunmüller and Unden, 2009), however, the complete absence of ABC transporters or PTS systems is unprecedented. Heterofermentative hexose metabolism via the phosphoketolase pathway yields only one mole of ATP per mole of glucose (Gänzle, 2015). Monosaccharide transport via ABC transporters or PTS systems thus consumes all of the metabolic energy yield that is generated through subsequent catabolism while oligosaccharide transport through secondary carriers is more efficient, particularly when coupled to disaccharide phosphorylases and the use of external electron acceptors (Gänzle, 2015) (Figure 3-4 above). Studies determining

the specificity of oligosaccharide carriers remain scarce but current evidence suggests that oligosaccharide transport remains limited to di-, tri- and tetra-saccharides (Gänzle and Follador, 2012; Hachem et al., 2013).

We identified the substrate specificity of the secondary transporters by a multi-pronged approach including bioinformatic analyses, comparison to biochemically characterized homologues, the genetic organization, and the effect of putative substrates on gene expression. A comparable approach previously identified genes coding for carbohydrate transport in *Bifidobacterium bifidum* (Turroni et al., 2012). Carriers for maltose, maltose- and isomaltose-oligosaccharides, sucrose, and lactose/ raffinose were highly expressed during growth of *L. reuteri* in sourdough and in the rodent forestomach (Figure 3-2 above) (Schwab et al., 2014). LacS-mediated lactose transport in *Streptococcus thermophilus* is inhibited by melibiose, suggesting that α-GOS and raffinose-family oligosaccharides are an additional substrate for LacS (Gänzle and Follador, 2012; Poolman et al., 1992) this conforms to the pattern of gene expression observed in this study (Figure 3-3 above). The sucrose transporter ScrT in *L. reuteri* is overexpressed by sucrose and raffinose, and is part of a sucrose utilization operon (Figure 3-3 above, and Figure 3-5 below) (Teixeira et al., 2013). The maltose transporter MalT in *L. reuteri* is induced by maltose. The maltose-utilization operon also encodes for maltose phosphorylase, phosphoglucomutase, and the regulator *malR* in addition to MalT.

3.4.3 Homofermentative and heterofermentative lifestyles–complement or competition?

Homofermentative and heterofermentative lactobacilli differ fundamentally with respect to metabolic pathways for carbohydrate uptake and metabolism, and with respect to the regulation of sugar metabolism (this study; Gänzle, 2015; Gänzle et al., 2007). Homofermentative and heterofermentative lactobacilli, however, co-exist in many intestinal and man-made habitats including the upper intestine of rodents, birds, and swine, insect intestinal microbiota, and cereal and dairy fermentations (Duar et al., 2017c; Gänzle et al., 2007; Zheng et al., 2015a). For example, microbiota of back-slopped sourdoughs contains homofermentative and heterofermentative lactobacilli (De Vuyst et al., 2014). In rice vinegar microbiota, heterofermentative *L. fermentum* is associated with the homofermentative *L. plantarum* and *L. casei* during the alcoholic fermentation stage (Wu et al., 2012). Co-existence of homofermentative and heterofermentative lactic acid bacteria was described as "resource partitioning" where one group of organisms preferentially utilizes glucose while the other preferentially utilizes oligosaccharides including maltose, sucrose, and raffinose (Gänzle et al., 2007; Schwab et al., 2014; Tannock et al., 2012). Our study on carbohydrate transport and metabolism in *L. reuteri* provides a molecular explanation for the differential preference for glucose and oligosaccharides in homofermentative and heterofermentative lactobacilli, and hence the co-existence of these organisms in many of their natural habitats and in food fermentations. This study also adds to the body of evidence that

a differentiation of the diverse genus *Lactobacillus* is required to appreciate its contribution to intestinal and food-associated ecosystems (Duar et al., 2017c; Zheng et al., 2015a).

In conclusion, this study provides a detailed physiological and genetic analysis of carbohydrate metabolism in the heterofermentative model species *L. reuteri*. The specificity of several predicted secondary carriers for carbohydrates was validated by quantification of gene expression in sourdough and in the chemically defined media. Our analysis revealed significant differences between *L. reuteri* and homofermentative lactobacilli, representing general differences between homofermentative and heterofermentative lactobacilli. The study contributes to our understanding of the co-existence of different lactobacilli in food fermentations on the molecular level and may be used to improve the assessment of the impact of lactobacilli on food quality.

3.5 References

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3.6 Supplementary materials

Table 3-5 Growth of *L. reuteri* in chemically defined medium with defined substrates after 48 h

Sugars	LTH 2584	TMW 1.112	TMW 1.656	LTH 5448	100-23	lpuph	mlc3
glycerol	-	-	-	-	-	-	-
erythritol	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-
L-arabinose	-	+	-	+	+	+	+
D-ribose	-	+	+	+	+	+	+
D-xylose	+	-	-	-	+	-	+
L-xylose	-	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-	-
Methyl-βD-xylopyranose	-	-	-	-	-	-	-
D-galactose	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+
D-fructose	-	-	-	-	-	-	-
D-mannose	-	-	-	-	-	-	-
L-sorbose	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-
Methyl-αD-Mannopyranose	-	-	-	-	-	-	-
Methyl-αD-Glucopyranose	+	+	+	+	+	+	-
N-acetylGlucosamine	-	-	-	-	-	-	-
amygdalin	-	-	-	-	-	-	-
arbutin	-	-	-	-	-	-	-
esculin	-	-	-	-	-	-	-
ferric citrate	-	-	-	-	-	-	-
salicin	-	-	-	-	-	-	-
D-cellobiose	-	-	-	-	-	-	-
D-maltose	+	+	+	+	+	+	+
D-lactose	+	+	+	+	+	+	+
D-melibiose	+	+	+	+	+	+	+
D- sucrose	+	+	+	+	+	+	+
D-trehalose	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	-
D-raffinose	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-

Table 3-5 Growth of *L. reuteri* in chemically defined medium with defined substrates after 48 h

Sugars	LTH 2584	TMW 1.112	TMW 1.656	LTH 5448	100-23	lpuph	mlc3
Gentiobiose	-	-	-	-	-	-	-
D-turanose	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-
K gluconate	+	+	-	+	+	-	+
K 2-ketoGluconate	-	-	-	-	-	-	-
K 5-ketoGluconate	-	-	-	-	-	-	-

Table 3-6 API ZYM enzyme profile for *L. reuteri* strains

Enzymes	LTH2584	TMW1.112	TMW1.656	LTH5448	100-23	mlc3	lpuph
Alkaline phophatase	-	-	-	-	-	-	-
Esterase(C4)	+	+	+	+	+	+	+
Esterase lipase (C8)	+	+	+	+	+	+	+
Lipase(C14)	-	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+	+	+
Crystine arylamidase	-	-	-	-	-	-	-
Trypsin	-	-	-	-	-	-	-
α-chymotrypsin	-	-	-	-	-	-	-
Acid phosphatase	+	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+
α-galactosidase	+	+	+	+	+	+	+
β-galactosidase	+	+	+	+	+	+	+
β-glucuronidase	-	-	-	-	-	-	-
α-glucosidase	+	+	+	+	+	+	+
β-glucosidase	-	-	-	-	-	-	-
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-

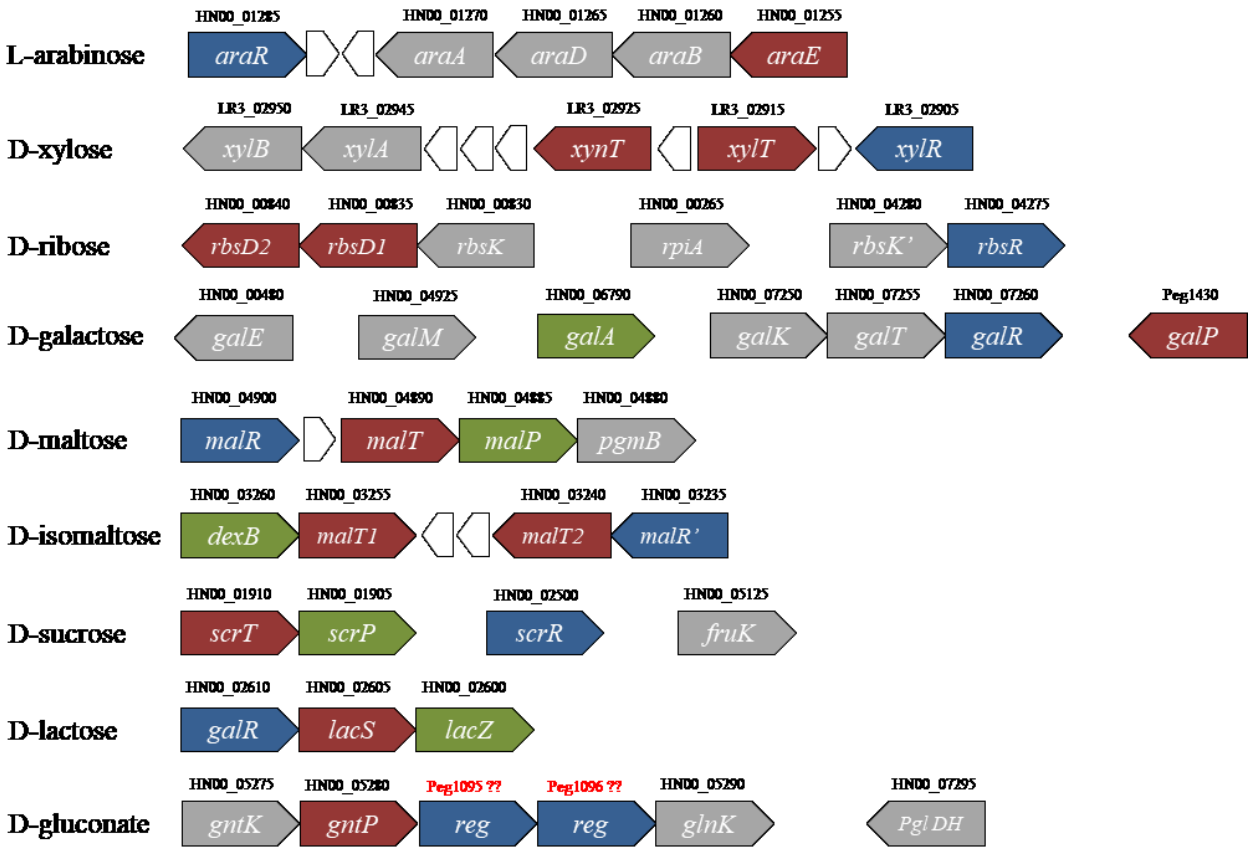

Figure 3-5 Representation of conserved gene cluster architectures for particular sugars metabolism in sourdough- and rodent- *L. reuteri*

Arrow direction indicates forward (left arrow) or reverse (right arrow) strand where ORFs are located. If two genes are adjacent to each other, they are connected in line without blank; if two neighbor genes are not adjacent but close to each other in chromosome, a blank distance would show in between; if two neighbor genes are far away from each other, they are connected with dash lines. D-xylose gene cluster are from the representative strain LTH2584, other clusters depicted above from strain LTH5448.
(1) **L-arabinose cluster**: ***araR***: transcriptional repressor of arabinoside utilization operon, GntR family; ***araA***: L-arabinose isomerase; ***araD***: L-ribulose-5-phosphate 4-epimerase; ***araB***: ribulokinase; ***araE***: arabinose-proton symporter. **(2)** **D-xylose cluster**: ***xylR***: xylose-responsive

transcription regulator, ROK family; ***xylT***: D-xylose proton-symporter; ***xynT***: xyloside transporter; ***xylA***: xylose isomerase; ***xylB***: xylulose kinase; **(3) D-ribose cluster**: ***rbsD2***: homolog of fucose/glucose/galactose permeases; ***rbsD1***: ribose ABC transport system, high affinity permease; ***rbsK***: ribokinase; ***rpiK***: ribose 5-phosphate isomerase A; ***rbsK***: ribokinase; ***rbsR***: ribose operon repressor. **(4) D-galactose cluster**: ***galE***: UDP-glucose 4-epimerase; ***galM***: aldose 1-epimerase; ***galA***: alpha-galactosidase; ***galK***: galactokinase; ***galT***: galactose-1-phosphate uridylyltransferase; ***galR***: galactose operon repressor, GalR-LacI family of transcriptional regulators; ***lacS***: lactose and galactose permease, GPH translocator family. **(5) D-maltose cluster**: ***malR***: maltose operon transcriptional repressor MalR, LacI family; ***malT***: maltose transporter; ***malP***: maltose phosphorylase; ***pgmB***: beta-phosphoglucomutase. **(6) D-isomaltose cluster**: ***malL***: oligo-1,6-glucosidase; ***malT***: sugar transport protein; ***malT2***: sugar transporter; ***malR***: transcriptional regulator, LacI family. **(7) D-sucrose cluster**: ***scrT***: sucrose transporter; ***scrP***: sucrose phosphorylase; ***scrR***: sucrose operon repressor ScrR, LacI family; ***gftA***: reuteransucrase. **(8) D-lactose cluster**: ***galR***: galactose operon repressor, GalR-LacI family of transcriptional regulators; ***lacS***: lactose and galactose permease, GPH translocator family; ***lacZ***: beta-galactosidase. **(9) D-gluconate cluster**: ***gntP***: gluconate permease; ***glnK***: gluconokinase; ***pglDH***: 6-phosphogluconate dehydrogenase; ***reg***: transcriptional regulator, LacI family

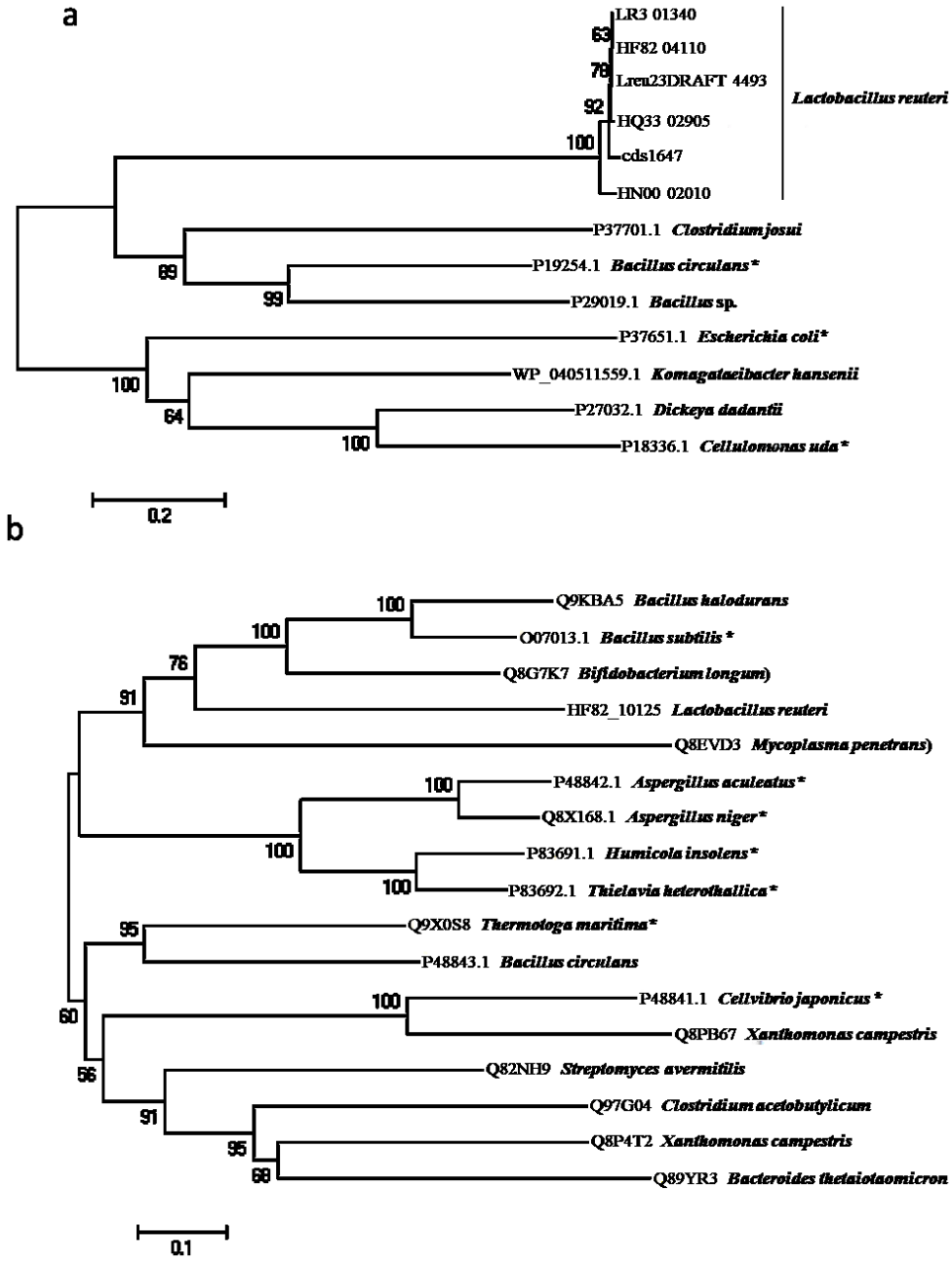

Figure 3-6 Unrooted phylogenetic trees of lichenase and arabinogalactan endo-β-1,4-galactanase of *L. reuteri* and related proteins

The trees were based on protein sequence alignment of the glycosyl hydrolase (GH) family domain in of lichenase (**a**) and arabinogalactan endo-β-1,4-galactanase (**b**). Proteins were selected from the Pfam domain seed alignment defining GH8 family and GH53 family. Proteins indicated with asterisks were characterized biochemically. Protein sequence alignment was represented as the Neighbor-Joining tree with bootstrap value of 500 iterations.

CHAPTER 4 Comparative genomics of *Lactobacillus reuteri* from sourdough reveals adaptation of an intestinal symbiont to food fermentations

4.1 Introduction

Lactobacillus reuteri persist in intestinal microbiota of vertebrate animals as well as in food fermentations (Gänzle, 2015; Oh et al., 2010; Vogel et al., 1999; Walter, 2008). *L. reuteri* colonizes humans and animal hosts (Oh et al., 2010; Walter, 2008); the phylogenetic differentiation of strains of *L. reuteri* originating from different hosts reflects co-evolution of *L. reuteri* with its vertebrate hosts (Oh et al., 2010). This evolutionary adaptation differentiates the species *L. reuteri* in host-adapted phylogenetic lineages comprised of isolates from rodents (lineages I and III), humans (lineages II and VI), pigs (lineages IV and V), and poultry (lineage VI) (Oh et al., 2010; Spinler et al., 2014).

L. reuteri also occur in industrial sourdoughs (Gänzle and Vogel, 2003) and cereal fermentations in tropical climates (Gänzle, 2015; Sekwati-Monang and Gänzle, 2011). Sourdoughs are typically maintained by continuous propagation, a process which rapidly selects for the most competitive microbiota. Major selection criteria for fermentation microbiota in cereal ecosystems are rapid growth in cereal substrates, and acid resistance (Böcker et al., 1995; Gänzle, 2015; Lin and Gänzle, 2014b; Meroth et al., 2003). Food isolates of *L. reuteri* match to host-adapted lineages (Su et al., 2012) and maintain host-specific physiological traits (Frese et al., 2013; Su and Gänzle, 2014; Wilson et al., 2014), including the ability to colonize the lineage-specific hosts (Frese et al., 2011; Su et al., 2012).

The differentiation of *L. reuteri* into host-adapted lineages implies that an extra-intestinal habitat did not exist for a majority of the evolution of this species (Frese et al., 2013). However, the occurrence of *L. reuteri* in the human-made habitat sourdough provides the opportunity to study the "reverse adaptation" of vertebrate symbionts to an extra-intestinal habitat. This study employed comparative genomics of *L. reuteri* to evaluate the genetic determinants of this adaptation or selection process. Genome sequences of intestinal strains of *L. reuteri* were retrieved from public databases and compared to four genome sequences of rodent-lineage sourdough isolates (Lin et al., 2015). The sourdough isolates *L. reuteri* LTH2584, TMW1.112 and TMW1.656 originate from SER sourdough, a sourdough that is used industrially for production of a baking improver (Böcker et al., 1995). This sourdough has been maintained by continuous propagation since about 1970. *L. reuteri* LTH2584, TMW1.1112 and TMW1.656 were isolated from this sourdough in 1988, 1994, and 1998 (Böcker et al., 1995; Gänzle and Vogel, 2003); all of these strains produce reutericyclin, a tetramic acid derivative with antimicrobial activity against Gram-positive bacteria (Gänzle and Vogel, 2003; Lin et al., 2015). *L. reuteri* LTH5448 was isolated from a different sourdough processed at the same facility in 2000 (Meroth et al., 2003; Schwab and Gänzle, 2006); this strain does not produce reutericyclin but maintains the reutericyclin genomic island and reutericyclin resistance (Lin et al., 2015; Schwab and Gänzle, 2006). Comparative genomics analyses included analyses of the core genome as well as gene gain and gene loss events that were studied on the basis of the pan-genome. We also performed positive selection analysis for these core genes of the whole species. Finally, the competitiveness of sourdough isolates of *L. reuteri* in model sourdoughs was compared to the competitiveness of closely related intestinal isolates.

4.2 Materials and Methods

4.2.1 Strains, media and growth conditions

The sourdough isolates *L. reuteri* LTH2584, TMW1.112, TMW1.656 and LTH5448 (Böcker et al., 1995; Gänzle and Vogel, 2003; Schwab and Gänzle, 2006) and the rodent isolates *L. reuteri* 100-23, mlc3, and lpuph (Wesney and Tannock, 1979) were grown anaerobically at 37°C in mMRS (Meroth et al., 2003). Sugars were autoclaved separately. Solid media contained additional 20 g agar per liter.

4.2.2 Whole-genome alignment and phylogenetics[1]

Genome sequences of the 12 *L. reuteri* were retrieved from Genbank (Table 4-4 below). Genome sequences of sourdough isolates (Lin et al., 2015) were re-annotated on the RAST server (Aziz et al., 2008) after gap closing by PCR amplification and Sanger sequencing. Primers binding to up- and down- stream locus of the target gap were selected after alignment of the genomes with Mauve (Darling et al., 2004), and are shown in Table 4-4 below. Sequencing was performed by service of Macrogen Co. (Rockville, Maryland, USA).

All 16 genomes were aligned with Mugsy (Angiuoli and Salzberg, 2011). Homologous blocks present in each genome were concatenated with an in-house perl script. The most disordered regions were eliminated using Gblocks (Talavera and Castresana, 2007). The disordered regions include sites containing at least one gap, and sites that are too divergent as they may not be homologous or may be saturated by multiple substitutions. The core genome size of *L. reuteri* was about 1.2 Mbp. A maximum-likelihood core genome tree was constructed using RaxML (Talavera

[1] Contribution by Dr. Jinshui Zheng

and Castresana, 2007). The tree was inferred under the general time-reversible nucleotide substitution model (GTR), with gamma-distributed rate heterogeneity of four rate categories (+Γ4) (Γ4). Bootstrap support values were calculated from 1000 replicates.

4.2.3 Gene clustering and construction of a gene content tree[2]

Protein sequences longer than 50 amino acids from all genomes were combined and searched using BLAST with an all-against-all style with default parameters. The protein sequences with identities and coverage above 70% were clustered into families using the program orthoMCL (Talavera and Castresana, 2007). The inflation value of 2 was used for the MCL clustering. Core genes were defined as those shared by all of the 16 strains; distributed genes as those shared by 2 to 15 strains, and unique genes as those only contained in one strain.

A matrix of the presence or absence of each gene for each genome was created. A dissimilarity distance between genomes based on gene content (binary data for presence or absence of each protein family) measured by one minus the Jaccard coefficient (Jaccard distance) was calculated from this matrix (Wolf et al., 2002). A gene content tree was constructed using the hierarchical clustering (UPGMA) method based on these distances by MEGA (Tamura et al., 2013).

4.2.4 Analysis of positive selection[3]

For each cluster of the single-copy core genes, protein sequences were aligned with MUSCLE (Edgar, 2004). These alignments were reverse-translated to codon-based nucleotide alignments

[2] Contribution by Dr. Jinshui Zheng

[3] Contribution by Dr. Jinshui Zheng

by PAL2NAL (Suyama et al., 2006). Positive selection analysis based on each of these alignments was performed by CODEML implemented in PAML (Yang, 2007).

Nonsynonymous (amino acid altering) synonymous (silent) substitution ratios (ω), with ω = 1, < 1, or > 1 indicate neutral, purifying, or positive selection, respectively. Positive selection was analyzed on each family of core genes shared by all the 16 *L. reuteri* isolates using the site models M1a and M2a (Wong et al., 2004; Yang et al., 2005). The model M1a (nearly neutral) allows all sites to be purifying selection ($\omega_0 < 1$) or neutral selection ($\omega_0 = 1$); the model M2a allows all sites to be positive selection ($\omega_0 > 1$). A likelihood ratio test (LRT) was carried out to infer the occurrence of sites subject to positive selective pressure through comparing M1a against M2a. Branch-site model and the one-ratio null model were used to analyze positive selection across the *L. reuteri* LTH2584/TWM1.112/TWM1.656 branch. Branch-site model allows ω to vary both among sites in the protein and across branches on the tree and aim to detect positive selection affecting a few sites along particular lineages (called foreground branches) (Zhang et al., 2005). Two models were used, the null model does not allow positive selection for the foreground branch, and the alternative model assumes that the foreground branch may have some sites under positive selection. For the alternative model, three classes of ω (dN/dS) were defined: $\omega_0 < 1$, $\omega_1 = 1$ and $\omega_2 \geqslant 1$, while in the null model, ω_2 was fixed to 1. A likelihood ratio test (LRT) was carried out to infer positive selective pressure across the *L. reuteri* LTH2584/TWM1.112/TWM1.656 branch through comparing the results from these two models. The LRT statistic (twice the log-likelihood difference between the null and the alternative models) was compared with the chi-square distribution with 2 degrees of freedom for M2a vs. M1a, and one degree of freedom for branch-site model *vs.* the null model.

For Clusters of Orthologous Groups of proteins (COG) analysis, we constructed a local COG database (Tatusov et al., 2003), and then ran rpsblast using the sequence sets mentioned above as queries. We focused on the top three hits of each alignment and counted each category for comparison using in-house Perl script.

4.2.5 Competitiveness of *L. reuteri* in sourdough: experimental design

The persistence of strains was analyzed in back-slopped rye sourdough fermentations; experiments were carried out with fermentation times of 1, 2, and 3 days. Competition experiments were carried out with six strain combinations; sourdoughs were inoculated with *L. reuteri* LTH2584 and 100-23; *L. reuteri* LTH5448 and 100-23; *L. reuteri* LTH5448 and mlc3; *L. reuteri* LTH5448 and lpuph; *L. reuteri* LTH2584 and LTH5448; or *L. reuteri* LTH2584, TMW1.112, TMW1.656, and LTH5448.

4.2.6 Sourdough preparation and differential enumeration of cell counts

Competition experiments in sourdough were performed essentially as described (Lin and Gänzle, 2014b). In brief, sourdough was prepared by mixing 10 g rye flour with 10 mL of autoclaved tap water and 1 mL of bacterial inoculum. For binary and quaternary strain combinations, 0.5 and 0.25 mL, respectively, of the cell suspensions of individual strains were mixed to obtain 1 mL of bacterial cocktail as inoculum. Dough was fermented at 37°C for 1, 2, or 3 days and back-slopped over 10 fermentation cycles.

At each back-slopping step, 1 g of ripe sourdough from the previous cycle was mixed with 9.5 g of fresh rye flour and 9.5 mL of autoclaved tap water. The competition experiments were performed in duplicate and analyses were carried out with two technical replicates. At each fermentation cycle, sourdoughs were analyzed with respect to the pH, differential cell counts, and qPCR with strain specific primers. Viable cell counts were enumerated by surface-plating of

appropriate dilutions on mMRS agar. Individual strains were differentiated on the basis of the colony morphology. Differential enumeration was possible for the binary strain combinations *L. reuteri* LTH2584 vs. 100-23, *L. reuteri* LTH2584 vs. LTH5448, *L. reuteri* LTH5448 vs. mlc3, and *L. reuteri* LTH5448 vs. lpuph, but not for *L. reuteri* LTH5448 vs. 100-23. In the quaternary strain combination, the combined total of *L. reuteri* LTH2584, TMW1.112 and TMW1.656 was differentiated from *L. reuteri* LTH5448.

4.2.7 Analysis of sourdough microbiota by qPCR

Total DNA was isolated from sourdough (Lin and Gänzle, 2014a) and gene copy numbers of L. reuteri were quantified by strain-specific qPCR. Strain-specific primers are listed in Table 4-1 below. Standard curves to convert detection threshold cycles to gene copy numbers were established by analysis of 10-fold serial dilutions of target DNA of known concentration.

Table 4-1 Primers used for the strain-specific PCR quantification of strains of *L. reuteri* in sourdoughs

Target strain		Primer (5'-3)	Annealing temp. (°C)	Amplicon size (bp)
LTH2584	84F1[a]	GGCGTTCCTTTAACTGCTTTAAC	58	95
	84R1	CTTCCTGTCCCACCAGAAATAA		
	84F2	TCTACCGGGTCTATGGCTATC	58	105
	84R2	CGTTGGGCAGGGTGTAAATA		
LTH5448	48F	TAAGGCTGCTCGCAAGTATTTA	58	131
	48R	CGGTATTTGCTTTCGCACTAAC		
100-23	23F	CCTTCATCAGTCTTAGCGTCTT	60	109
	23R	GCGATAGCTGGAATGGGATTA		
TMW1.112	12F	TTGTTGTCGTTGGTGGTATGA	60	103
	12R	CCTCCAACTGCTAAACCAATCT		
TMW1.656[b]	56F	GCAGCCCAAGTAACTGAAGA	58	132
	56R	CCACCAACCAGGAAGCATAA		
	wF	GGCGGAACGTTGAATATTGT	58	248
	wR	ATTTTGGGGGAATCATAGCC		
TMW1.656Δ*rtcN*Δ*rtcT*[b]	mF	CACGTGTGTCAATAAAAAGCTGA	58	156
	mR	AACTAAACGTGCCCCATTTG		

[a]The primer pair 84F1/R1 was used for differentiating of *L. reuteri* LTH2584 from strains *L. reuteri* TMW1.112 and TMW1.656; the primer pair 84F2/R2 was used for differentiation from *L. reuteri* 100-23.
[b]The primer pairs wF/wR and mF/mR were used for quantification of *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*rtcN*Δ*rtcT*, respectively, in competition experiments with the wild type and mutant strains.

Calibration curves to convert gene copy numbers to cell counts in sourdough were established with sourdoughs that were fermented with single strains. Samples were mixed with 2 volumes of sterile saline, and serially diluted with saline. From each of the dilutions, cell counts were determined by further dilution and surface plating and the gene copy numbers were quantified by qPCR as described above. Calibration curves were established in duplicate after 1 and 3 d of fermentation (Figure 4-8 below).

Quantitative PCR analyses were carried out in duplicate in MicroAmp Fast Optical 96-well reaction plates capped with MicroAmp Optical Adhesive Film (Applied Biosystems, Burlington, ON, Canada). The PCR reaction mixture consisted of 12.5 µL Fast SYBR Green Master Mix (Applied Biosystems), 0.4 µM of each primer (Table 4-3 below), 2 µL of template DNA and sterile

Milli-Q water to final volume of 25 µl. Melting curves were obtained by a stepwise increase of the temperature from 60 to 95°C at 0.05°C/s and melting-curve data were analyzed to verify amplification of the correct targeted PCR products. The detection limit was 10^2 copy numbers/g sourdough for the strain-specific primers.

4.2.8 Competitiveness of isogenic reutericyclin-positive and reutericyclin-negative and reutericyclin-sensitive isogenic strains of *L. reuteri*[4]

Competition experiment between reutericyclin-positive wild type strain *L. reuteri* TMW1.656 and its reutericyclin-susceptible mutant L. reuteri TMW1.656Δ*rtcN*Δ*rtcT* (Lin et al., 2015) were performed using white wheat flour with a dough yield of 200. Sourdough was propagated every 24h with 1% inoculum for 5 days. The ratio of wild type to mutant strains at the end of each fermentation cycle was determined by qPCR with primers listed in Table 4-1 above.

4.2.9 Calculation of the relative fitness of strains of *L. reuteri* in sourdough

The differential cell counts and the strain-specific gene copy numbers were used to calculate the relative fitness of the respective strains of *L. reuteri*. The fitness (w) of strain x relative to that of strain y was calculated based an equation derived from (Kerr et al., 2002):

$$w(\mathrm{x},\mathrm{y}) = \ln \frac{x_F/x_o}{y_F/y_o}$$

Where x_0 and y_0 denote the strain specific cell densities or gene copy numbers at the beginning of each fermentation cycle and x_F and y_F are cell densities at the end of each of fermentation cycle.

[4] Contribution by Dr. Xiaoxi B. Lin

For each competition experiment, the relative fitness was plotted as average of 20 replicates (replicate experiments with 10 fermentation cycles each).

4.3 Results

4.3.1 Phylogenetic analysis of 16 sequenced *L. reuteri* strains including 4 sourdough strains[5]

The phylogenetic analysis was carried out with all available genome sequences of *L. reuteri*, including 4 genome sequences of sourdough isolates (Lin et al., 2015). A phylogenetic tree was constructed based on the core genome of *L. reuteri* (Figure 4-1A below). Strains of *L. reuteri* were grouped into 5 clusters corresponding to the host-adapted lineages I (rodent), II (human), III (rodent), IV (pig) and VI (poultry and human). Sourdough strains were assigned to the rodent-adapted lineages I and III, in agreement with previous analyses (Su et al., 2012). *L. reuteri* LTH5448 clustered with lineage I rodent isolates; *L. reuteri* LTH2584, TWM1.112 and TWM1.656 were grouped into lineage III together with the rodent isolates *L. reuteri* 100-23 and mlc3. *L. reuteri* LTH2584, an SER sourdough isolate obtained in 1988, was more closely related to *L. reuteri* TWM1.656, which was isolated from SER sourdough in 1998, than to *L. reuteri* TWM1.112, which was isolated from the same sourdough in 1994 (Gänzle and Vogel, 2003).

A gene content tree was constructed to study the gain and loss of genes among these strains. Here, strains sharing more genes were clustered together (Figure 4-1B below). The topology of the gene content tree was different from the core genome phylogenetic tree, indicating gene loss or acquisition of genes by horizontal genetic transfer. Three clusters corresponding to linages II, IV

[5] Contribution by Dr. Jinshui Zheng

and VI were maintained but the gene content tree highlighted differences between strains in each cluster. For example, the four lineage II *L. reuteri* MM4-1A, MM2-3, DSM 20016 and JCM 1112 were not separated in the core genome phylogenetic tree but differentiated in two groups by calculating the gene content tree (Figure 4-1B below). *L. reuteri* DSM20016 and JCM1112 were derived from the same original isolate, F275, and differences between these two strains may reflect loss of genes during propagation in the laboratory (Morita et al., 2008). The two lineage III strains *L. reuteri* 100-23 and mlc3 showed a quite different gene content. Remarkably, all four sourdough isolates were grouped together despite their divergent phylogenetic origin. *L. reuteri* LTH5448 was more closely related to *L. reuteri* LTH2584 than to *L. reuteri* TWM1.112 and TWM1.656.

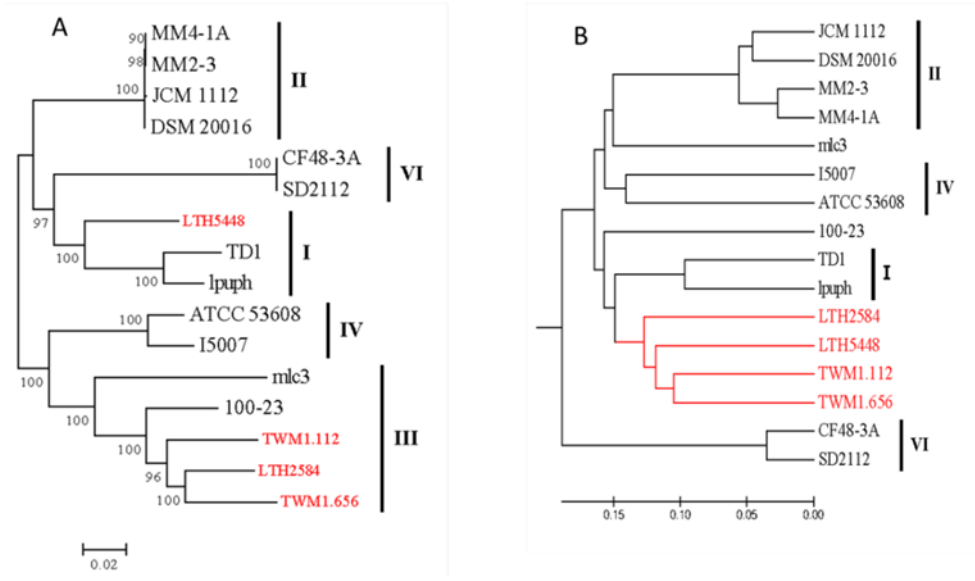

Figure 4-1 Phylogenetic analysis of the 16 *L. reuteri* strains[6]

(**A**) Phylogenetic tree based on core genes. Roman numerals designate the host adapted lineages of *L. reuteri*. Strains isolates from sourdough are marked in red. Only bootstrap values above 90 were shown. Branch length are proportional to the number of substitutions per site (see scale bar). Note that this tree does not imply specific amounts of time per branch, nor does it indicate when particular branching events occurred. (**B**) Genome tree based on gene content matrix. Scale bar represent the genetic distance as determined by Jaccard distance.

4.3.2 Comparative analysis of sourdough strains

To understand how the intestinal strains adapted to sourdough, and to identify genes that are unique to sourdough isolates, the gene content similarity and dissimilarity of these strains was analyzed. *L. reuteri* LTH2584, TWM1.112, TWM1.656 and 100-23 shared 1535 core genes (Figure 4-2A below); this core genome is higher than the core genome of the whole species (Spinler et al., 2014), reflecting that all these strains are grouped in the lineage III. *L. reuteri* LTH2584 and 100-23 had more unique genes than *L. reuteri* TWM1.112 and TWM1.656 (Figure 4-2A below), which contributed to the distinct position of the former two strains in the gene content tree. Sourdough isolates shared 1523 core genes (Figure 4-2B below).

[6] Contribution by Dr. Jinshui Zheng

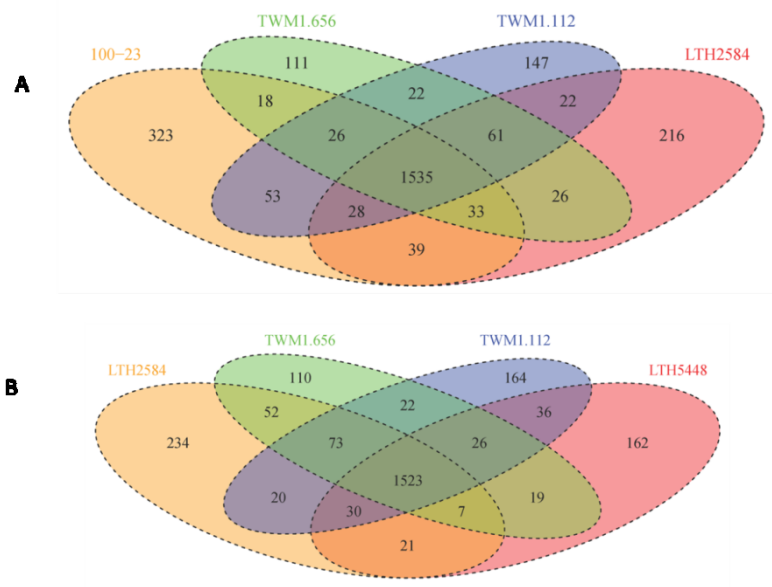

Figure 4-2 Comparative analyses between sourdough isolates and 100-23 and among sourdough isolates[7]

(**A**) Venn diagram of core, distributed and unique gene numbers among Lineage III strains LTH2584, TMW1.656, TMW1.112 and 100-23. (**B**) Venn diagram of core, distributed and unique gene numbers among the sourdough isolates in Lineage III, LTH2584, TMW1.656, TMW1.112, and Lineage I sourdough isolate *L. reuteri* LTH5448.

Genes that were shared by all sourdough isolates but absent in other strains include the chromosomally encoded reutericyclin genomic island (Lin et al., 2015), a putative aspartate racemase, a LytTr-domain protein with putative regulatory function, and components of a putative ABC-transporter (Table 4-2 below). Genes that were only present in some of the sourdough isolates include a glycosyltransferases with putative function in protein glycosylation (LTH2584 and TMW1.656) and a putative hydroxyglutarate dehydrogenase (LTH2584 and TMW1.112) which catalyses the use of α-ketoglutarate as electron acceptor (Zhang and Gänzle, 2010). Of note, distributed genes that are present in sourdough isolates of *L. reuteri* and other strains include several putative enzymes of the shikimic acid pathway for biosynthesis of aromatic amino acids

[7] Contribution by Dr. Jinshui Zheng

(Table 4-2 below). In summary, only genes coding for reutericyclin biosynthesis are unique to all sourdough isolates of *L. reuteri*.

Table 4-2 Distributed genes specific to sourdough strains[8]

Gene or gene cluster	(Putative) function
Exclusive to all sourdough strains (*L. reuteri* LTH2584, TMW1.112, TMW1.656, and LTH5448)	
Reutericyclin genomic island	Reutericyclin biosynthesis and resistance (Lin et al., 2015)
Components of an ABC transporter	Unknown (Lin et al., 2015)
Aspartate racemase (WP_003670574.1)	
LytTr DNA-binding domain (WP_006729038.1)	Unknown
Exclusive to some sourdough strains	
***L. reuteri* LTH2584 and TMW1.112**	
GntR (WP_006916030.1)	
Membrane transport protein (WP_006916028.1)	Unknown
Hydroxyglutarate dehydrogenase (WP_006916027.1)	Use of ketoglutarate as electron acceptor (Zhang and Gänzle, 2010)
***L. reuteri* LTH2584 and TMW1.656**	
Two GT8_A4GalT_like proteins (WP_020807748.1, WP_020807748.1)	Broad spectrum glycosyltransferases with putative function in protein glycosylation
YkuD (WP_003664366.1)	Peptidoglycan crosslinking
Predominantly sourdough strains	
***L. reuteri* TMW1.112, TMW1.656 and mlc3**	
mlc3|WP_019251925.1, WP_019251926.1, WP_019251927.1, WP_019251928.1; WP_019251930.1; WP_019251931.1; WP_019251932.1; WP_019251933.1	Putative components of shikimic acid pathway for biosynthesis of aromatic amino acids
***L. reuteri* TMW1.112, LTH5448 and 100-23**	
Homocysteine methyltransferase (100_23|ZP_03072304)	
S-methylmethionine transporter (100_23|ZP_03072305)	Oxidative stress response
***L. reuteri* LTH2584, TMW1.112, TMW1.656, and 100-23**	
Lr100-23|ZP_03073418.1|	NADPH-dependent FMN reductase. rRNA
Lr100-23|ZP_03073416.1|	Methyltransferase
Lr100-23|ZP_03073414.1|	Lipid metabolism
Lr100-23|ZP_03073413.1|	Serine protease
Lr100-23|ZP_03073412.1|	Metal-dependent beta-lactamase superfamily I

[8] Contribution by Dr. Jinshui Zheng

Table 4-2 Distributed genes specific to sourdough strains[8]

Gene or gene cluster	(Putative) function
Lr100-23|ZP_03073411.1|	Unknown
Lr100-23|ZP_03073410.1|	Unknown
Lr100-23|ZP_03073409.1|	Histidine kinase
Lr100-23|ZP_03073446.1|	Cardiolipin synthase
Lr100-23|ZP_03073445.1|	Acetyltransferase (GNAT) family

Note: Hypothetical proteins and phage-related proteins were excluded from the list. Protein numbers refer to the genome of *L. reuteri* LTH2584 unless otherwise specified.

4.3.3 Positive selection of the core genes contributing to the adaptation of sourdough isolates

Analysis of positive selection aimed to identify the selective pressure on the core genome of *L. reuteri*, and to determine whether sourdough and intestinal strains are subjected to a differential selective pressure. Initially, positive election was analyzed in all 16 strains of *L. reuteri*. A total of 124 core genes were under positive selection (Figure 4-3 below, and Table 4-5 below), representing 10.36% of the core genome. Among the genes that are under positive selection, 22% relate to metabolism, including transporters and enzymes for protein, amino acid, carbohydrate, and lipid conversion. Several genes under positive selection were listed as "general functional prediction only", but most of these predicted functions were also related to metabolic functions. Other abundant genes under positive selection relate to DNA replication, recombination, and repair.

When compared with the composition of the core genes, COG categories "translation, ribosomal structure and biogenesis" and "general functional prediction only" were significantly enriched among genes under positive selection in all 16 core genomes of *L. reuteri* ($P = 0.04, 0.03$, one-sided binomial test). For the 20 genes in the former category, 8 are tRNA associated genes, 6 are ribosomal protein genes, 3 are 23S RNA-specific pseudouridylate synthases, 2 are translation

elongation factor genes, and 1 is methylase of polypeptide chain release factor gene (Table 4-5 below). For the latter category, most predicted functions relate to metabolism. For example, 6 out 21 were hydrolases, and other were some reductases, permeases and esterases.

To identify the selective pressure acting on the sourdough isolates, the branch-site model and its null model were used to compare the function categories under positive selection in the branch comprising *L. reuteri* LTH2584, TWM1.112, and TWM1.656 to all other strains in the species. A total of 177 core genes were under positive selection in these lineage III sourdough isolates (Figure 4-3 below, and Table 4-6 below). Of these, 135 genes were under positive selection only in this branch and the remaining 42 were under positive selection in the sourdough isolates as well as the remainder of the species. Of the core genes under positive selection in the sourdough branch, 33% related to metabolism (Figure 4-3 below, and Table 4-6 below). Three COG categories were significant enriched in the lineage III sourdough isolates, “Energy production and conversion” ($P = 5.9$), “Carbohydrate transport and metabolism” ($P = 0.03$) and “Defense mechanisms” ($P < 2.2$) (Figure 4-3 below). Examples of gene in these COG categories that are under positive selection include key metabolic enzymes such as maltose phosphorylase, lactate dehydrogenase, alcohol dehydrogenase, and several sugar transport enzymes (Table 4-6 below).

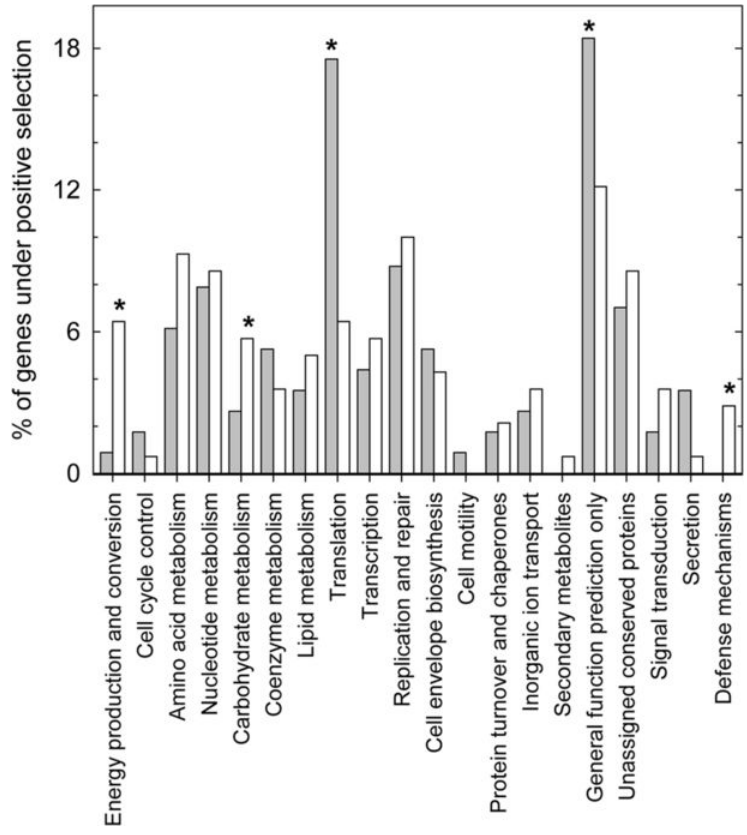

Figure 4-3 Proportions of positive selection of core genes in COG categories in *L. reuteri* [9]

Proportions of positively selected genes in COG categories for all *L. reuteri* isolates (gray bars), sourdough isolates in Lineage III comprising *L. reuteri* LTH2584, TWM1.112, and TWM1.656 (white bars). Site model M2a and its null model M1a were compared to infer genes under positive selection in the whole species. Branch-site mode and its null model were compared to study genes under positive selection across the COG categories. COG categories that were significantly enriched are marked by an asterisk.

4.3.4 Competitiveness of *L. reuteri* strains in sourdough: experimental design

To determine whether genomic adaptation of *L. reuteri* to the sourdough environment increases the competitiveness of strains, competition experiments sourdoughs were carried out. Competition experiments in back-slopped sourdoughs are a sensitive tool to determine the competitiveness of

[9] Contribution by Dr. Jinshui Zheng

strains because even small differences in competitiveness result in predominance of the more competitive strain after few refreshments (Lin and Gänzle, 2014b; Su et al., 2011). Experiments were performed with fermentation cycles of 1, 2, or 3 days. The selection of strains used in the competition experiments included the sourdough isolates *L. reuteri* LTH5448, LTH2584, TMW1.112 and TMW1.656; and the rodent isolates *L. reuteri* 100-23, mlc3 and lpuph. Two methods were used to achieve strain specific quantification of *L. reuteri* in sourdough. When used in combination, qPCR and differential plate counts ensured that sourdough microbiota in all samples consisted of only of those strains used as inoculum, and accurately quantified the strain specific contribution to the fermentation microbiota.

The competition experiments were evaluated by calculation of the relative fitness. An example for the evaluation of competition experiments by qPCR and differential viable cell counts is shown in Figure 4-4 below; all experiments are shown in Figure 4-9 below of the online supplementary material. The cell counts and the pH of sourdough were influenced by the fermentation time and the fermentation microbiota. Three day fermentation cycles results in lower cell counts and higher pH values at the end of the fermentation when the glutamate-decarboxylase positive *L. reuteri* LTH5448 was part of the fermentation microbiota (Figure 4-10 below, and Figure 4-11 below).

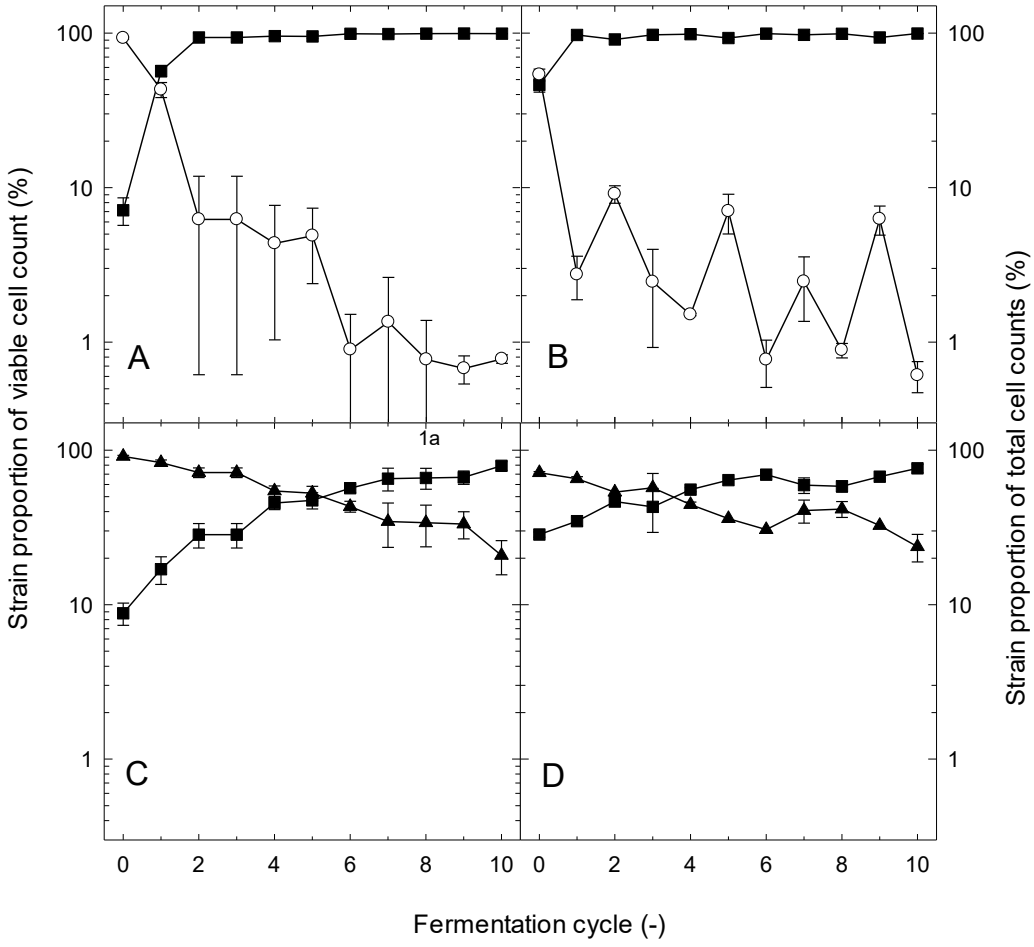

Figure 4-4 Evolution of fermentation microbiota in binary strain competitions of strains of *L. reuteri*

Sourdoughs were inoculated with *L. reuteri* LTH2584 and 100-23 (**Panels A and B**) or with *L. reuteri* LTH2584 and LTH5448 (**Panels C and D**) and maintained by continuous back-slopping with 10% inoculum over 10 fermentation cycles with 24h incubation times. Sourdough microbiota were analyzed by differential plate counts **(Panels A and C)** and results were expressed as log proportion of the individual strains to the total viable cell counts. Sourdoughs were also analyzed by qPCR targeting strain-specific sequences and log DNA copy numbers converted to cell counts using the strain-specific calibration curves (**Panels B and D**). Results were expressed as proportion of the individual strains to the total cell counts. Symbols represent *L. reuteri* strains LTH2584 (■), LTH5448 (▲) and 100-23 (○).

The competitiveness of the sourdough isolates *L. reuteri* LTH2584 and LTH5448 was substantially higher when compared to the rodent isolate *L. reuteri* 100-23 (Figure 4-5 below). This difference in competitiveness to the reutericyclin-sensitive *L. reuteri* 100-23 was equivalent for the reutericyclin-producing *L. reuteri* LTH2584 and the reutericyclin-negative *L. reuteri*

LTH5448. To determine whether this increased competitiveness was influenced by reutericyclin-production, a competition experiment was carried out between the reutericyclin-producing *L. reuteri* TMW1.656 and the isogenic reutericyclin-negative and -sensitive *L. reuteri* TMW1.656Δ*rtcN*Δ*rtcT* (Lin et al., 2015).

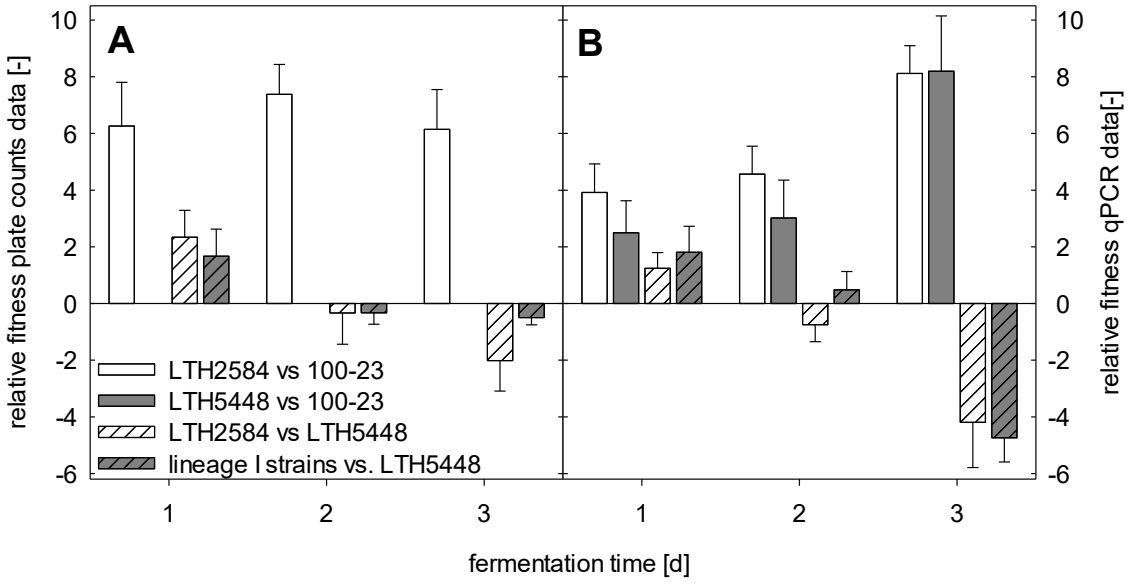

Figure 4-5 Relative fitness of strains of *L. reuteri* in sourdoughs back-slopped with 1d, 2d, or 3d fermentation cycles

The relative fitness of strains was calculated from differential cell counts **(Panel A)** and from qPCR with strain specific primers **(Panel B)** by using the equation: $w(x,y) = \ln \frac{x_F / x_0}{y_F / y_0}$, where x, y denote the strains used in binary strain combinations, x_F and y_F denote the cell counts or gene copy numbers at the end of each fermentation cycle and x_0 and y_0 denote the cell counts or gene copy numbers at the beginning of each fermentation cycle. The relative fitness was calculated for the following strain combinations: *L. reuteri* LTH2584 versus 100-23 (white bars); *L. reuteri* LTH5448 versus 100-23 (gray bars); *L. reuteri* LTH2584 versus LTH5448 (white hatched); and lineage III strains consisting of *L. reuteri* LTH2584, TMW1.112 and TMW1.656 versus lineage I strain *L. reuteri* LTH5448 (gray hatched). Differential enumeration was not possible for the strain combination *L. reuteri* LTH5448 and 100-23, therefore, only qPCR data is shown for this strain combination. Each competition was performed in doughs with 10 fermentation cycles that were back-slopped every 1, 2 and 3 days per fermentation cycle, respectively. The data was expressed as mean ± standard deviation from experiments performed in duplicate with two technical replicates.

L. reuteri TMW1.656 and TMW1.656Δ*rtcN*Δ*rtcT* exhibited a comparable competitiveness and were maintained in approximately equal cell counts over 6 fermentation cycles (Figure 4-6 below). This result demonstrates that reutericyclin production does not substantially influence the competitiveness of *L. reuteri* strains in sourdough.

Competition experiments between the sourdoughs isolates of *L. reuteri* revealed that all strains that were used as inoculum were maintained over 10 fermentation cycles (Figure 4-9 below). The competitiveness of the sourdough strains reflected the effects of lineage-specific metabolic traits on competitiveness in sourdough (Lin and Gänzle, 2014b; Su et al., 2011). Glutamate decarboxylase mediates acid resistance; presence of this enzyme also increases competitiveness in sourdoughs with a long fermentation time (Lin and Gänzle, 2014b). Accordingly, the glutamate-decarboxylase-negative lineage III strains were more competitive in sourdoughs that were maintained by short fermentation cycles while the glutamate-decarboxylase positive lineage I strain *L. reuteri* LTH5448 was more competitive in sourdoughs that were maintained with 2 and 3 d cycles (Figure 4-5 above).

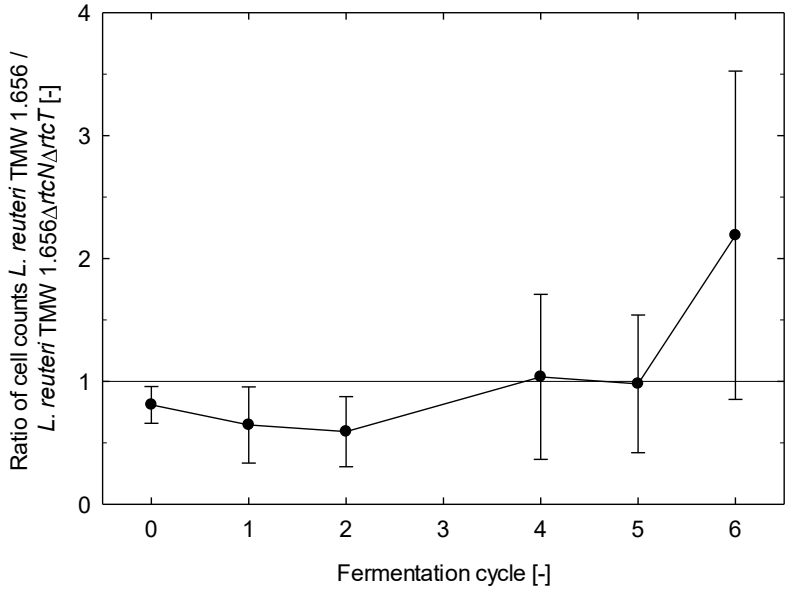

Figure 4-6 Evolution of fermentation microbiota in sourdoughs inoculated with the reutericyclin producing wild type strain *L. reuteri* TMW1.656 and the isogenic reutericyclin-negative and reutericyclin-sensitive *L. reuteri* TMW1.656Δ*rtcN*Δ*rtcT*[10]

Evolution of fermentation microbiota in sourdoughs inoculated with the reutericyclin producing wild type strain *L. reuteri* TMW1.656 and the isogenic reutericyclin-negative and reutericyclin-sensitive *L. reuteri* TMW1.656Δ*rtcN*Δ*rtcT* (Lin et al., 2015). The ratio of wild type strain to mutant strain was determined by qPCR analysis with strain-specific primers. Results are shown as means ± standard deviation of triplicate independent experiments.

4.4 Discussion

This study employed comparative genomics to demonstrate that the evolution of *L. reuteri* is shaped by positive selection of the core genome in addition to the gain and loss of accessory genes. Moreover, the identification of core genes under positive selection and the analysis of competitiveness in sourdough demonstrate that sourdough isolates of *L. reuteri* have adapted to the new habitat, or have been selected from a distinct subset of rodent lineage strains. This study

[10] Contribution by Dr. Xiaoxi B. Lin

provides new insights into the adaptation of *L. reuteri* to food and intestinal habitats, suggesting that these two habitats exert different selective pressure related to growth rate and metabolism.

4.4.1 Core genome phylogenetic analysis confirms host-specific lineages

Core genome phylogenetic analysis of the 16 strains of *L. reuteri* confirmed differentiation into host-specific lineages (Su et al., 2012). The rodent lineage III strains *L. reuteri* LTH2584, TWM1.112, and TWM1.656 were isolated form the same sourdough in 1988, 1994, and 1998, respectively. The phylogenetic relatedness of these strains suggested that the later isolates may be isolates of the same organism after 10 years of adaptation to sourdough fermentation (Gänzle and Vogel, 2003). However, successive contamination of the same sourdough with different strains of rodent origin is an alternative explanation for the isolation of highly related strains from the same sourdough.

4.4.2 Role of reutericyclin production for competitiveness in sourdoughs

Reutericyclin production may contribute to competitiveness of *L. reuteri* in sourdough (Gänzle and Vogel, 2003; Vogel et al., 1999). This study demonstrated that a reutericyclin sensitive derivative of *L. reuteri* TMW1.656 and the reutericyclin producing wild type strain exhibited comparable competitiveness in sourdough, indicating that the ecological advantage of reutericyclin is about equivalent to the cost of reutericyclin production (Abrudan et al., 2012; Lin et al., 2015). The reutericyclin gene cluster was acquired by horizontal gene transfer by few Lineage I and Lineage III sourdough isolates of *L. reuteri* (Gänzle and Vogel, 2003; Lin et al., 2015) and is thus unlikely to represent a sourdough-specific metabolic trait.

4.4.3 Evolution of the intestinal symbiont *L. reuteri* by horizontal gene transfer and positive selection

The evolution of pathogens is driven by gene loss, acquisition of genes by horizontal gene transfer, and by positive selection of the core genome (Lefébure and Stanhope, 2007, 2009; Orsi et al., 2008). The relative contribution of recombination and positive selection are highly dependent on the species and the ecosystem. Only few genes were reported to be under positive selective pressure in the pathogenic *Listeria monocytogenes* (Orsi et al., 2008) while up to 34% and 92%, respectively, of the core genome were positively selected in specific lineages of the host-adapted genera *Streptococcus* and *Campylobacter* (Lefébure and Stanhope, 2007, 2009). The evolution of the host adapted gut symbiont *L. reuteri* was previously attributed to gene loss and acquisition of lineage-specific accessory genes (Frese et al., 2011). The congruent clustering of *L. reuteri* strains in the phylogenetic tree and the gene content tree confirms a major role of the gain or loss of host-specific metabolic and genetic traits in the evolution of the species (Frese et al., 2011, 2013). This study additionally demonstrates that positive selection of the core genome shapes the evolution of *L. reuteri*. The expression of ribosomal proteins, rRNA and other transcription factors is regulated by the bacterial growth rate (Klumpp et al., 2009) and a high density of ribosomal genes relates to rapid growth of *L. sanfranciscensis* in sourdough (Vogel et al., 2011). Positive selection in the functional category translation indicates that the intestinal ecological niches harboring *L. reuteri* exert selective pressure for rapid growth. The proportion of the core genome that was under positive selection in *L. reuteri* matches the corresponding proportion in *Streptococcus mutans* (Lefébure and Stanhope, 2007). *S. mutans* and *L. reuteri* are phylogenetically related host-adapted organisms colonizing the upper intestinal tract but contrast with respect to their impact on the hosts. *S. mutant* is a pathogen and *L. reuteri* is considered as probiotic but both species apparently

use comparable ecological strategies for colonization and persistence (Gänzle and Schwab, 2009; Lin et al., 2015).

4.4.4 Reverse evolution or selection of *L. reuteri* in an extra-intestinal habitat?

The persistence of *L. reuteri* over 10 years in sourdough fermentation provides a unique opportunity to study the adaptation or selection of a host-specific gut symbiont to an extra-intestinal environment. Several lines of evidence suggest that SER sourdough isolates are distinct from intestinal strains. First, sourdough isolates cluster separately in the gene content tree, indicating that horizontal gene transfer and the loss of genes relates to the transition to sourdough (Lin et al., 2015). Second, the functional categories "energy production and conversion" and "carbohydrate metabolism", which are key elements for competitiveness in sourdough (Gänzle et al., 2007; Passerini et al., 2013), were significantly enriched among the positively selected genes in SER isolates. Third, sourdough isolates of *L. reuteri* displayed a higher relative fitness in sourdough when compared to rodent isolates. *L. reuteri* LTH5448 also achieved a high proportion of cell counts in competition with rodent isolates *L. reuteri* mlc3 and lpuph in experiments with 2 d fermentation time (data not shown). The differences in competitiveness of rodent and sourdough isolates, however, are smaller than differences between individual strains, reflecting the relatedness of the rodent forestomach and sourdough environments (Schwab et al., 2014). Because the time between sourdough contamination with *L. reuteri* and the isolation of the specific strains is unknown, it is not possible to discriminate whether the specific differences of the sourdough isolates reflect selection for a specific subset of rodent isolates, or "reverse evolution" of a gut symbiont to food fermentations.

In conclusion, this study demonstrated that gene loss and gene gain as well as selective pressure on the core genome drive the evolution of *L. reuteri*. Remarkably, the gene content of sourdough

isolates of *L. reuteri* differed from intestinal isolates, and genes under positive selection in sourdough strains included maltose phosphorylase, alcohol dehydrogenase, and lactate dehydrogenase, genes which are known to contribute to competitiveness in cereal fermentations. The study improves our understanding of the adaptation of bacteria to food fermentations as an evolutionary recent man-made habitat. It will also improve our ability to use food fermentations as model systems for more complex, intestinal ecosystems (Wolfe and Dutton, 2015).

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4.6 Supplementary materials

Table 4-3 Primers for closing of gaps in the whole genome shotgun sequences of *L. reuteri* TMW1.112 and *L. reuteri* TMW1.656

Gap within scaffold	Primers (5'-3')(forward / reverse)	Elongation time (sec)
***L. reuteri* TMW1.112**		
Gap A1+A2	For: CACACCAAATTCGCGTTC Rev: TGCTTAATGCGTTAGCTC	220
Gap A3	For: TACCCGAAAGTAGTCGAG Rev: CTTCCGCAAAATAGGTGT	150
Gap A4	For: CTTCCGCAAAATAGGTGT Rev: GATGGTGCAGTCTTGGG	150
Gap A5	For: CTTTAGCACTCCGCATT Rev: AAGAACGTGGTAATGCTC	90
Gap A6	For: ATTCGGGTCCTCAACCAC Rev: GCTGACCGCTTGTCGAG	90
Gap A14	For: AGGTTATGCTTACGGTCA Rev: ACGGAAGTTCGACGTTG	90
Gap A15+A16+A17	For: ATTTTCCACAGCCCGTTG Rev: TCCAAAATTAACCGCTCT	220
Gap A18+19	For: CTGCGAATTAAATTGGTC Rev: CGCAAATATCTGTACCCT	120
***L. reuteri* TMW1.656**		
Gap A1	For: TAGTCGCCAATAATCGAG Rev: TGCATCATTTAAGCACCT	260
Gap A3	For: AACCAAATCAGCGAGCAG Rev: CAGCCCATTGAAGGGTT	120
Gap A4	For: TGAAACGGTAATGCAAG Rev: GCACGATCATAATTGGG	220
Gap A7	For: CATAATGATATAGCCCTGT Rev: CAACGTGGACTAGAACCC	180
Gap A8	For: TGGAAATAGTTGCCCGTA Rev: TAATTATGCGGTGACTGG	160

Table 4-4 Genome characteristics of strains used in this study[11]

Strain number	Accession number	# of contigs
DSM 20016	NC_009513.1	1
JCM 1112	NC_010609.1	1
SD2112	NC_015697.1; NC_015700.1; NC_015698.1; NC_015701.1; NC_015699.1	1 chromosome, 4 plasmids
I5007	NC_021494.1; NC_021503.1; NC_021495.1; NC_021496.1; NC_021497.1; NC_021504.1; NC_021498.1	1 chromosome, 6 plasmids
TD1	NC_021872.1	1
CF48-3A	NZ_ACHG00000000.1	92
MM2-3	NZ_ACLB00000000.1	95
ATCC 53608	NZ_CACS00000000.2	64
MM4-1A	NZ_ACGX00000000.2	7
100-23	NZ_AAPZ00000000.2	2
mlc3	NZ_AEAW00000000.1	126
Lpuph	NZ_AEAX00000000.1	127
LTH2484	NZ_JOSX00000000.1	25
TMW1.656	NZ_JOSW00000000.1	24
TMW1.112	NZ_JOKX00000000.1	48
LTH5448	NZ_JOOG00000000.1	36

[11] Contribution by Dr. Jinshui Zheng

Table 4-5 Genes under positive selection in the 16 genomes of *L. reuteri*[12]

Protein Acc. No in 100-23	COG	COG category	Function
WP_003666558.1	COG0356	C	F0F1-type ATP synthase, subunit a
WP_003665654.1	COG0037	D	tRNA(Ile)-lysidine synthase MesJ
WP_003666765.1	COG0849	D	Cell division ATPase FtsA
WP_003665400.1	COG1296	E	Predicted branched-chain amino acid permease (azaleucine resistance)
WP_003666360.1	COG0263	E	Glutamate 5-kinase
WP_003666409.1	COG0624	E	Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases
WP_003666512.1	COG0078	E	Ornithine carbamoyltransferase
WP_003666614.1	COG0028	EH	Thiamine pyrophosphate-requiring enzymes
WP_003666411.1	COG0834	ET	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain
WP_003665376.1	COG1957	F	Inosine-uridine nucleoside N-ribohydrolase
WP_003665434.1	COG0046	F	Phosphoribosylformylglycinamidine (FGAM) synthase, synthetase domain
WP_003666321.1	COG0209	F	Ribonucleotide reductase, alpha subunit
WP_003665438.1	COG0150	F	Phosphoribosylaminoimidazole (AIR) synthetase
WP_003666060.1	COG0207	F	Thymidylate synthase
WP_003665127.1	COG0518	F	GMP synthase - Glutamine amidotransferase domain
WP_003665407.1	COG0044	F	Dihydroorotase and related cyclic amidohydrolases
WP_003666124.1	COG1957	F	Inosine-uridine nucleoside N-ribohydrolase
WP_003664237.1	COG2814	G	Arabinose efflux permease
WP_003666072.1	COG2017	G	Galactose mutarotase and related enzymes
WP_003665682.1	COG0061	G	NAD kinase
WP_003663649.1	COG0697	GER	Permeases of the drug/metabolite transporter (DMT) superfamily
WP_003663861.1	COG0142	H	Geranylgeranyl pyrophosphate synthase
WP_003664178.1	COG0294	H	Dihydropteroate synthase and related enzymes
WP_003664181.1	COG0285	H	Folylpolyglutamate synthase
WP_003664112.1	COG0237	H	Dephospho-CoA kinase
WP_003664185.1	COG0801	H	7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase
WP_003664677.1	COG0657	I	Esterase/lipase
WP_003663678.1	COG0825	I	Acetyl-CoA carboxylase alpha subunit
WP_003665328.1	COG0183	I	Acetyl-CoA acetyltransferase
WP_003663677.1	COG0777	I	Acetyl-CoA carboxylase beta subunit
WP_003664573.1	COG0480	J	Translation elongation factors (GTPases)
WP_003665683.1	COG0564	J	Pseudouridylate synthases, 23S RNA-specific
WP_003664908.1	COG0480	J	Translation elongation factors (GTPases)

[12] Contribution by Dr. Jinshui Zheng

Table 4-5 Genes under positive selection in the 16 genomes of *L. reuteri*[12]

Protein Acc. No in 100-23	COG	COG category	Function
WP_003664503.1	COG0721	J	Asp-tRNAAsn/Glu-tRNAGln amidotransferase C subunit
WP_003666543.1	COG2890	J	Methylase of polypeptide chain release factors
WP_003664494.1	COG2265	J	SAM-dependent methyltransferases related to tRNA (uracil-5-)-methyltransferase
WP_003664519.1	COG0101	J	Pseudouridylate synthase
WP_003665664.1	COG1190	J	Lysyl-tRNA synthetase (class II)
WP_003665361.1	COG2265	J	SAM-dependent methyltransferases related to tRNA (uracil-5-)-methyltransferase
WP_003666200.1	COG0564	J	Pseudouridylate synthases, 23S RNA-specific
WP_003666676.1	COG0343	J	Queuine/archaeosine tRNA-ribosyltransferase
WP_003665800.1	COG0858	J	Ribosome-binding factor A
WP_003665090.1	COG0441	J	Threonyl-tRNA synthetase
WP_003666455.1	COG1670	J	Acetyltransferases, including N-acetylases of ribosomal proteins
WP_003664575.1	COG0049	J	Ribosomal protein S7
WP_003664569.1	COG0051	J	Ribosomal protein S10
WP_003664006.1	COG0211	J	Ribosomal protein L27
WP_003666161.1	COG0144	J	tRNA and rRNA cytosine-C5-methylases
WP_003664517.1	COG0102	J	Ribosomal protein L13
WP_003665822.1	COG1490	J	D-Tyr-tRNAtyr deacylase
WP_003664993.1	COG1476	K	Predicted transcriptional regulators
WP_003664629.1	COG0789	K	Predicted transcriptional regulators
WP_003666175.1	COG1609	K	Transcriptional regulators
WP_003665403.1	COG0583	K	Transcriptional regulator
WP_003666533.1	COG1438	K	Arginine repressor
WP_003666847.1	COG0608	L	Single-stranded DNA-specific exonuclease
WP_003663796.1	COG0178	L	Excinuclease ATPase subunit
WP_003665002.1	COG2094	L	3-methyladenine DNA glycosylase
WP_003663571.1	COG0419	L	ATPase involved in DNA repair
WP_003666195.1	COG0116	L	Predicted N6-adenine-specific DNA methylase
WP_003664214.1	COG2818	L	3-methyladenine DNA glycosylase
WP_003663858.1	COG0497	L	ATPase involved in DNA repair
WP_003664638.1	COG1573	L	Uracil-DNA glycosylase
WP_003666672.1	COG0323	L	DNA mismatch repair enzyme (predicted ATPase)
WP_003666649.1	COG2003	L	DNA repair proteins
WP_003665027.1	COG0791	M	Cell wall-associated hydrolases (invasion-associated proteins)
WP_003666800.1	COG0791	M	Cell wall-associated hydrolases (invasion-associated proteins)
WP_003664439.1	COG5632	M	N-acetylmuramoyl-L-alanine amidase
WP_003665598.1	COG0766	M	UDP-N-acetylglucosamine enolpyruvyl transferase
WP_003665614.1	COG0770	M	UDP-N-acetylmuramyl pentapeptide synthase
WP_003666198.1	COG0597	MU	Lipoprotein signal peptidase

Table 4-5 Genes under positive selection in the 16 genomes of *L. reuteri*[12]

Protein Acc. No in 100-23	COG	COG category	Function
WP_003664205.1	COG1705	NU	Muramidase (flagellum-specific)
WP_003666352.1	COG1214	O	Inactive homolog of metal-dependent proteases, putative molecular chaperone
WP_003665807.1	COG0576	O	Molecular chaperone GrpE (heat shock protein)
WP_003664641.1	COG0471	P	Di- and tricarboxylate transporters
WP_003664764.1	COG1464	P	ABC-type metal ion transport system, periplasmic component/surface antigen
WP_003664880.1	COG1122	P	ABC-type cobalt transport system, ATPase component
WP_003664817.1	COG2262	R	GTPases
WP_003666191.1	COG3331	R	Penicillin-binding protein-related factor A, putative recombinase
WP_003664848.1	COG2936	R	Predicted acyl esterases
WP_003666665.1	COG1058	R	Predicted nucleotide-utilizing enzyme related to molybdopterin-biosynthesis enzyme MoeA
WP_003666828.1	COG2333	R	Predicted hydrolase (metallo-beta-lactamase superfamily)
WP_003663828.1	COG1461	R	Predicted kinase related to dihydroxyacetone kinase
WP_003666365.1	COG0488	R	ATPase components of ABC transporters
WP_003664976.1	COG0656	R	Aldo/keto reductases, related to diketogulonate reductase
WP_003666471.1	COG0561	R	Predicted hydrolases of the HAD superfamily
WP_003666484.1	COG0110	R	Acetyltransferase (isoleucine patch superfamily)
WP_003666380.1	COG1040	R	Predicted amidophosphoribosyltransferases
WP_003664926.1	COG0628	R	Predicted permease, member of the PurR regulon
WP_003665008.1	COG2249	R	Putative NADPH-quinone reductase (modulator of drug activity B)
WP_003666217.1	COG0456	R	Acetyltransferases
WP_003666258.1	COG2985	R	Predicted permease
WP_003666215.1	COG4814	R	Uncharacterized protein with an alpha/beta hydrolase fold
WP_003663772.1	COG0561	R	Predicted hydrolases of the HAD superfamily
WP_003663776.1	COG4814	R	Uncharacterized protein with an alpha/beta hydrolase fold
WP_003665364.1	COG0431	R	Predicted flavoprotein
WP_003664885.1	COG1073	R	Hydrolases of the alpha/beta superfamily
WP_003664349.1	COG4684	S	Predicted membrane protein
WP_003664422.1	COG2246	S	Predicted membrane protein
WP_003665129.1	COG4841	S	Uncharacterized protein conserved in bacteria
WP_003666456.1	COG2461	S	Uncharacterized conserved protein
WP_003664210.1	COG1598	S	Predicted nuclease of the RNAse H fold, HicB family
WP_003665003.1	COG3189	S	Uncharacterized conserved protein
WP_003666105.1	COG3152	S	Predicted membrane protein

Table 4-5 Genes under positive selection in the 16 genomes of *L. reuteri*[12]

Protein Acc. No in 100-23	COG	COG category	Function
WP_003664609.1	COG1814	S	Uncharacterized membrane protein
WP_003666679.1	COG1862	U	Preprotein translocase subunit YajC
WP_003663819.1	COG0552	U	Signal recognition particle GTPase
WP_003665010.1	-	-	hypothetical protein
WP_003664831.1	-	-	hypothetical protein
WP_003665115.1	-	-	hypothetical protein
WP_003664438.1	-	-	hypothetical protein
WP_003664432.1	-	-	hypothetical protein
WP_003664768.1	-	-	hypothetical protein
WP_003664021.1	-	-	hypothetical protein
WP_003666316.1	-	-	hypothetical protein
WP_003664935.1	-	-	hypothetical protein
WP_003666693.1	-	-	hypothetical protein
WP_003665064.1	-	-	hypothetical protein
WP_003664971.1	-	-	hypothetical protein
WP_003666804.1	-	-	hypothetical protein
WP_003665000.1	-	-	hypothetical protein
WP_003664232.1	-	-	hypothetical protein
WP_003666047.1	-	-	hypothetical protein
WP_003664174.1	-	-	hypothetical protein

Table 4-6 Genes under positive selection of the 3 lineage II sourdough isolates[13]

Protein Acc. Number in 100-23	COG	COG category [a]	Function
WP_003665061.1	COG0371	C	Glycerol dehydrogenase and related enzymes
WP_003666182.1	COG1304	C	Isopentenyl diphosphate isomerase (BS_ypgA, MTH48 and related proteins)
WP_003666317.1	COG1454	C	Alcohol dehydrogenase, class IV
WP_003666809.1	COG0022	C	Pyruvate/2-oxoglutarate dehydrogenase complex, dehydrogenase (E1) component, eukaryotic type, beta subunit
WP_003663509.1	COG0554	C	Glycerol kinase
WP_003665460.1	COG1757	C	Na+/H+ antiporter
WP_003664644.1	COG0281	C	Malic enzyme
WP_003664822.1	COG1052	CHR	Lactate dehydrogenase and related dehydrogenases
WP_003666646.1	COG4987	CO	ABC-type transport system involved in cytochrome bd biosynthesis, fused ATPase and permease components
WP_003666634.1	COG4477	D	Negative regulator of septation ring formation
WP_003665488.1	COG1174	E	ABC-type proline/glycine betaine transport systems, permease component
WP_003665217.1	COG0367	E	Asparagine synthase (glutamine-hydrolyzing)
WP_003666793.1	COG0289	E	Dihydrodipicolinate reductase
WP_003666790.1	COG2171	E	Tetrahydrodipicolinate N-succinyltransferase
WP_003666789.1	COG0019	E	Diaminopimelate decarboxylase
WP_003666795.1	COG0136	E	Aspartate-semialdehyde dehydrogenase
WP_003663503.1	COG4690	E	Dipeptidase
WP_003666788.1	COG0527	E	Aspartokinases
WP_003663799.1	COG0346	E	Lactoylglutathione lyase and related lyases
WP_003666203.1	COG0458	EF	Carbamoylphosphate synthase large subunit (split gene in MJ)
WP_003666792.1	COG0329	EM	Dihydrodipicolinate synthase/N-acetylneuraminate lyase
WP_003665343.1	COG0516	F	IMP dehydrogenase/GMP reductase

[13] Contribution by Dr. Jinshui Zheng

Table 4-6 Genes under positive selection of the 3 lineage II sourdough isolates[13]

Protein Acc. Number in 100-23	COG	COG category [a]	Function
WP_003664513.1	COG0503	F	Adenine/guanine phosphoribosyltransferases and related PRPP-binding proteins
WP_003665442.1	COG0138	F	AICAR transformylase/IMP cyclohydrolase PurH (only IMP cyclohydrolase domain in Aful)
WP_003665425.1	COG0026	F	Phosphoribosylaminoimidazole carboxylase (NCAIR synthetase)
WP_003664874.1	COG1957	F	Inosine-uridine nucleoside N-ribohydrolase
WP_003666778.1	COG0775	F	Nucleoside phosphorylase
WP_003664512.1	COG0026	F	Phosphoribosylaminoimidazole carboxylase (NCAIR synthetase)
WP_003664236.1	COG1328	F	Oxygen-sensitive ribonucleoside-triphosphate reductase
WP_003666048.1	COG0283	F	Cytidylate kinase
WP_003666825.1	COG2131	F	Deoxycytidylate deaminase
WP_003666786.1	COG0462	FE	Phosphoribosylpyrophosphate synthetase
WP_003666231.1	COG2814	G	Arabinose efflux permease
WP_003665600.1	COG1455	G	Phosphotransferase system cellobiose-specific component IIC
WP_003665332.1	COG1554	G	Trehalose and maltose hydrolases (possible phosphorylases)
WP_003666179.1	COG3345	G	Alpha-galactosidase
WP_003666129.1	COG2271	G	Sugar phosphate permease
WP_003666465.1	COG1109	G	Phosphomannomutase
WP_003666099.1	COG2610	GE	H+/gluconate symporter and related permeases
WP_003663565.1	COG4464	GM	Capsular polysaccharide biosynthesis protein
WP_003664212.1	COG0192	H	S-adenosylmethionine synthetase
WP_003664171.1	COG0351	H	Hydroxymethylpyrimidine/phosphomethyl pyrimidine kinase
WP_003666061.1	COG0262	H	Dihydrofolate reductase
WP_003663791.1	COG1154	HI	Deoxyxylulose-5-phosphate synthase
WP_003666183.1	COG1577	I	Mevalonate kinase
WP_003666184.1	COG3407	I	Mevalonate pyrophosphate decarboxylase
WP_003664987.1	COG1502	I	Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthases and related enzymes
WP_003666185.1	COG1577	I	Mevalonate kinase
WP_003664876.1	COG1028	IQR	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)

Table 4-6 Genes under positive selection of the 3 lineage II sourdough isolates[13]

Protein Acc. Number in 100-23	COG	COG category [a]	Function
WP_003666802.1	COG1597	IR	Sphingosine kinase and enzymes related to eukaryotic diacylglycerol kinase
WP_003664099.1	COG1534	J	Predicted RNA-binding protein containing KH domain, possibly ribosomal
WP_003664211.1	COG0495	J	Leucyl-tRNA synthetase
WP_003664555.1	COG0197	J	Ribosomal protein L16/L10E
WP_003666774.1	COG0060	J	Isoleucyl-tRNA synthetase
WP_003665453.1	COG0162	J	Tyrosyl-tRNA synthetase
WP_003664420.1	COG0024	J	Methionine aminopeptidase
WP_003663844.1	COG0144	J	tRNA and rRNA cytosine-C5-methylases
WP_003666836.1	COG0050	J	GTPases - translation elongation factors
WP_003665806.1	COG1420	K	Transcriptional regulator of heat shock gene
WP_003665864.1	COG0568	K	DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32)
WP_003665360.1	COG1609	K	Transcriptional regulators
WP_003663822.1	COG0571	K	dsRNA-specific ribonuclease
WP_003665379.1	COG1475	K	Stage 0 sporulation protein J (antagonist of Soj) containing ParB-like nuclease domain
WP_003663849.1	COG1198	L	Primosomal protein N' (replication factor Y) - superfamily II helicase
WP_003666046.1	COG0514	L	Superfamily II DNA helicase
WP_003666068.1	COG0164	L	Ribonuclease HII
WP_003666675.1	COG2255	L	Holliday junction resolvasome, helicase subunit
WP_003665794.1	COG2176	L	DNA polymerase III, alpha subunit (gram-positive type)
WP_003664114.1	COG0749	L	DNA polymerase I - 3'-5' exonuclease and polymerase domains
WP_003664109.1	COG3611	L	Replication initiation/membrane attachment protein
WP_003664108.1	COG1484	L	DNA replication protein
WP_003666331.1	COG0353	L	Recombinational DNA repair protein (RecF pathway)
WP_003666843.1	COG0322	L	Nuclease subunit of the excinuclease complex
WP_003666785.1	COG0507	L	ATP-dependent exoDNAse (exonuclease V), alpha subunit - helicase superfamily I member
WP_003665646.1	COG1197	LK	Transcription-repair coupling factor (superfamily II helicase)
WP_003664424.1	COG0513	LKJ	Superfamily II DNA and RNA helicases

Table 4-6 Genes under positive selection of the 3 lineage II sourdough isolates[13]

Protein Acc. Number in 100-23	COG	COG category [a]	Function
WP_003666070.1	COG0758	LU	Predicted Rossmann fold nucleotide-binding protein involved in DNA uptake
WP_003666313.1	COG1970	M	Large-conductance mechanosensitive channel
WP_003664075.1	COG0791	M	Cell wall-associated hydrolases (invasion-associated proteins)
WP_003665812.1	COG0481	M	Membrane GTPase LepA
WP_003664166.1	COG0744	M	Membrane carboxypeptidase (penicillin-binding protein)
WP_003666285.1	COG1066	O	Predicted ATP-dependent serine protease
WP_003665710.1	COG0450	O	Peroxiredoxin
WP_003665585.1	COG1108	P	ABC-type Mn2+/Zn2+ transport systems, permease components
WP_003665584.1	COG1121	P	ABC-type Mn/Zn transport systems, ATPase component
WP_003665505.1	COG1135	P	ABC-type metal ion transport system, ATPase component
WP_003664851.1	COG0783	P	DNA-binding ferritin-like protein (oxidative damage protectant)
WP_003664521.1	COG0619	P	ABC-type cobalt transport system, permease component CbiQ and related transporters
WP_003665028.1	COG2984	R	ABC-type uncharacterized transport system, periplasmic component
WP_003664082.1	COG1418	R	Predicted HD superfamily hydrolase
WP_003664101.1	COG1161	R	Predicted GTPases
WP_003664648.1	COG1064	R	Zn-dependent alcohol dehydrogenases
WP_003666059.1	COG0488	R	ATPase components of ABC transporters with duplicated ATPase domains
WP_003666235.1	COG0661	R	Predicted unusual protein kinase
WP_003664911.1	COG0596	R	Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily)
WP_003665414.1	COG0637	R	Predicted phosphatase/phosphohexomutase
WP_003665032.1	COG4120	R	ABC-type uncharacterized transport system, permease component
WP_003665591.1	COG1078	R	HD superfamily phosphohydrolases
WP_003665468.1	COG4989	R	Predicted oxidoreductase
WP_003666680.1	COG0618	R	Exopolyphosphatase-related proteins
WP_003665130.1	COG0656	R	Aldo/keto reductases, related to diketogulonate reductase
WP_003664635.1	COG0693	R	Putative intracellular protease/amidase
WP_003665035.1	COG1101	R	ABC-type uncharacterized transport system, ATPase component

Table 4-6 Genes under positive selection of the 3 lineage II sourdough isolates[13]

Protein Acc. Number in 100-23	COG	COG category [a]	Function
WP_003663793.1	COG3410	S	Uncharacterized conserved protein [Function unknown]
WP_003664639.1	COG2966	S	Uncharacterized conserved protein
WP_003666772.1	COG2302	S	Uncharacterized conserved protein, contains S4-like domain
WP_003666127.1	COG4485	S	Predicted membrane protein
WP_003664133.1	COG5584	S	Predicted small secreted protein
WP_003664998.1	COG3864	S	Uncharacterized protein conserved in bacteria
WP_003666192.1	COG4474	S	Uncharacterized protein conserved in bacteria
WP_003664634.1	COG2898	S	Uncharacterized conserved protein
WP_003664653.1	COG4320	S	Uncharacterized protein conserved in bacteria
WP_003663831.1	COG1302	S	Uncharacterized protein conserved in bacteria
WP_003664958.1	COG0586	S	Uncharacterized membrane-associated protein
WP_003665876.1	COG2996	S	Predicted RNA-bindining protein (contains S1 and HTH domains)
WP_003665610.1	COG0394	T	Protein-tyrosine-phosphatase
WP_003664953.1	COG0589	T	Universal stress protein UspA and related nucleotide-binding proteins
WP_003666821.1	COG3480	T	Predicted secreted protein containing a PDZ domain
WP_003666394.1	COG1493	T	Serine kinase of the HPr protein, regulates carbohydrate metabolism
WP_003665820.1	COG0317	TK	Guanosine polyphosphate pyrophosphohydrolases/synthetases
WP_003666531.1	COG1680	V	Beta-lactamase class C and other penicillin binding proteins
WP_003665254.1	COG1132	V	ABC-type multidrug transport system, ATPase and permease components
WP_003665082.1	COG1136	V	ABC-type antimicrobial peptide transport system, ATPase component
WP_003664219.1	COG4767	V	Glycopeptide antibiotics resistance protein
WP_003665469.1	-	-	hypothetical protein
WP_003666270.1	-	-	hypothetical protein
WP_003664842.1	-	-	hypothetical protein
WP_003665076.1	-	-	hypothetical protein
WP_003666708.1	-	-	hypothetical protein
WP_003666333.1	-	-	hypothetical protein
WP_003665706.1	-	-	hypothetical protein
WP_003664651.1	-	-	hypothetical protein

Table 4-6 Genes under positive selection of the 3 lineage II sourdough isolates[13]

Protein Acc. Number in 100-23	COG	COG category [a]	Function
WP_003665589.1	-	-	hypothetical protein

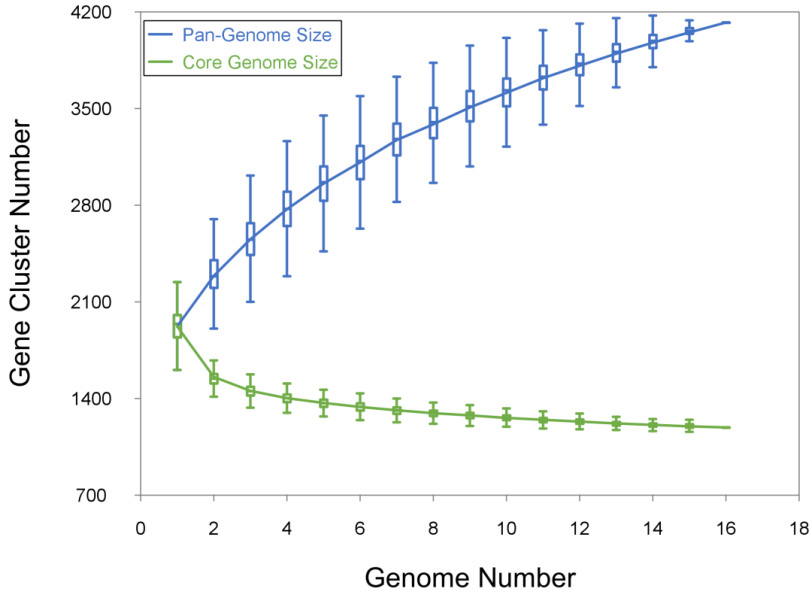

Figure 4-7 Visual representation of the core genome and the pan genome of *L. reuteri*[14]

The figure was generated with the PanGP software (http://sourceforge.net/projects/pangp/)

[14] Contribution by Dr. Jinshui Zheng

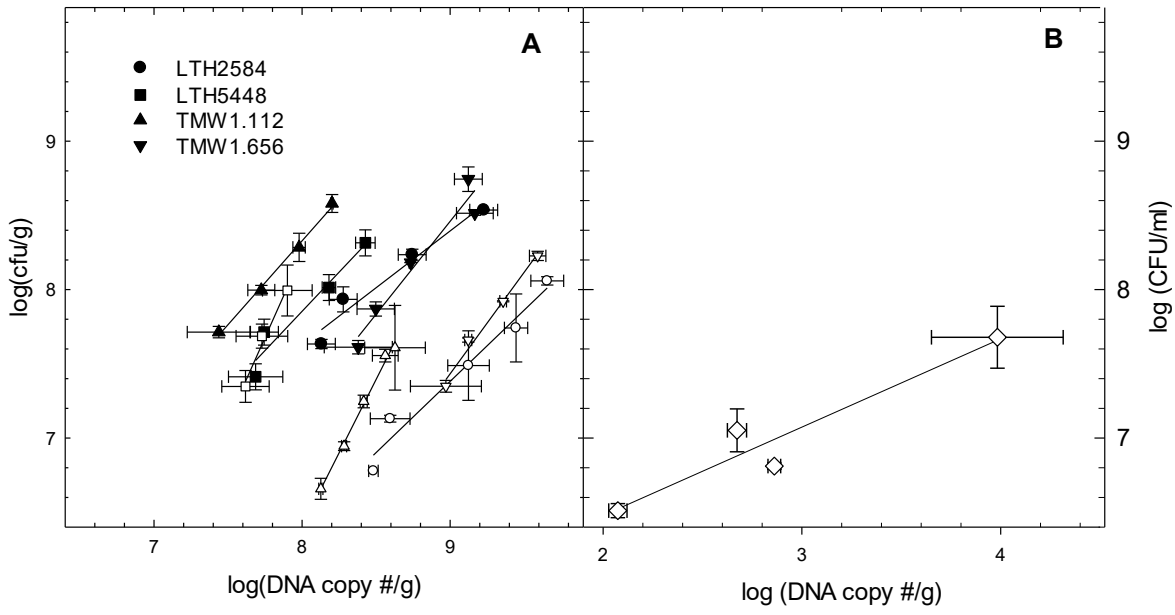

Figure 4-8 Calibration curves to convert qPCR data (gene copy numbers) to cell counts in rye sourdough

Calibration curves were established by analysis of sourdoughs after 12 h of fermentation (**black symbols)** or after 72 h of fermentation (**white symbols). Panel A:** *L. reuteri* LTH2584, LTH5448; TMW1.112 and TMW 1.656. **Panel B:** *L. reuteri* 100-23. Data are shown as means ± standard deviation of triplicate or quadruplicate independent experiments.

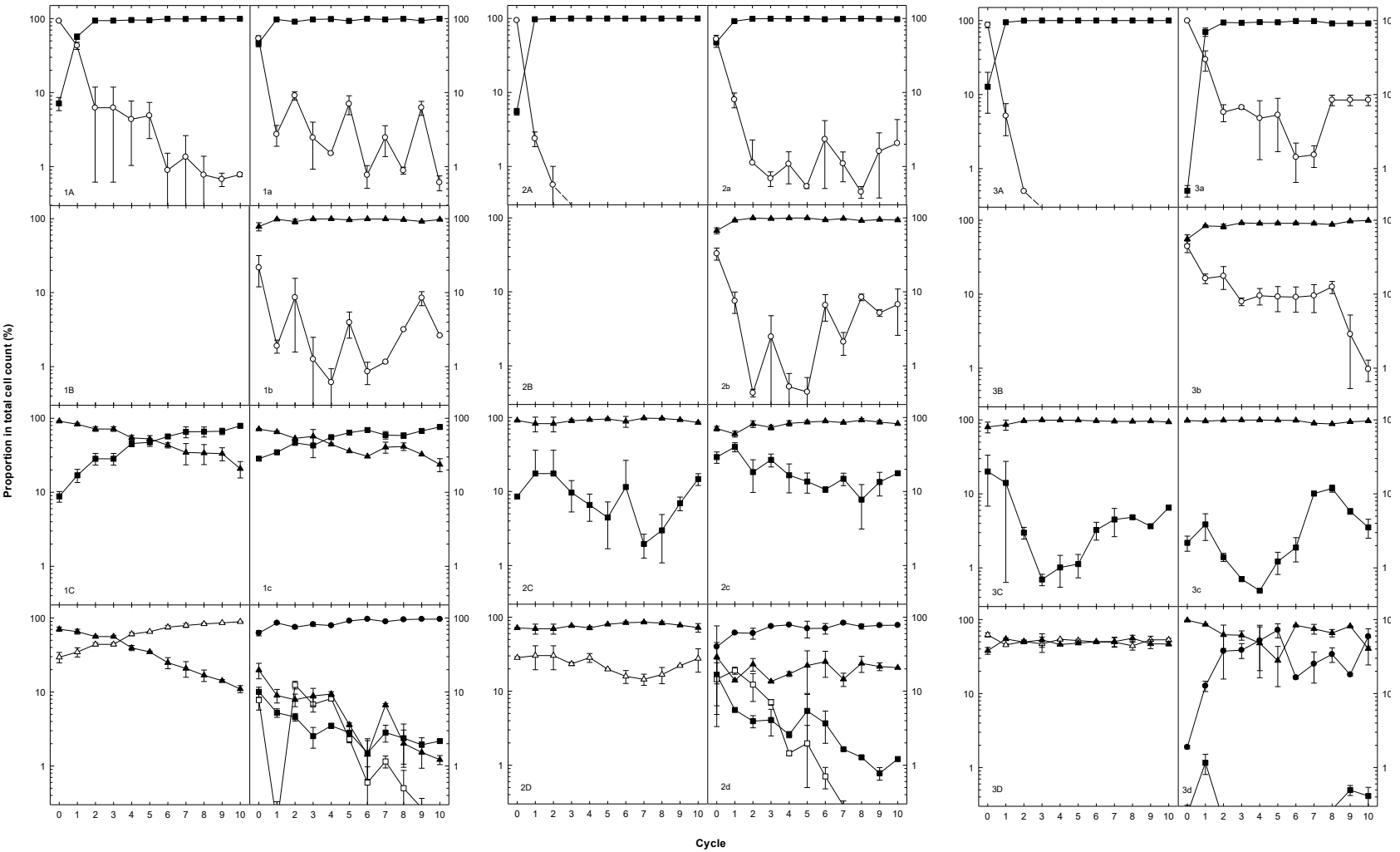

Figure 4-9 Cell density of individual *L. reuteri* isolates in competition experiments in sourdough

Evolution of fermentation microbiota in binary or quarternary strain competitions of strains of *L. reuteri*. Sourdoughs were inoculated with specific strains maintained by continuous back-slopping with 10% inoculum over 10 fermentation cycles with 1d, 2d, or 3d incubation times. Sourdough microbiota were analyzed by differential plate counts and results were expressed as log proportion of the individual strains to the total viable cell counts. Sourdoughs were also analyzed by qPCR targeting strain-specific sequences and log DNA copy numbers converted to cell counts using the strain-specific calibration curves and results were expressed as proportion of the individual strains to the total cell counts.

Panels are labeled with numbers and letters. The numbers indicate the fermentation time in days, capital letters indicate panels showing differential plate counts, and lowercase letters indicate panels showing results of strain specific qPCR analysis. Letters indicate the strains used in the competition as follows: A, a: *L. reuteri* LTH2584 (■) versus 100-23 (○); B, b: LTH5448 (▲) versus 100-23 (○); C, c: *L. reuteri* LTH2584 (■) versus LTH5448 (▲); D, d: *L. reuteri* LTH2584 (■) versus LTH5448 (▲), TMW1.656 (●) and TMW1.112 (□). The three lineage I strains *L. reuteri* LTH2584, TMW1.656 and TMW1.112 could not be selectively enumerated by differential plate counts, therefore the sum of all three strains is shown in panels depicting place count data (Δ). Data are shown expressed as mean ± standard deviation from two independent experiments with two technique replicates in each experiment.

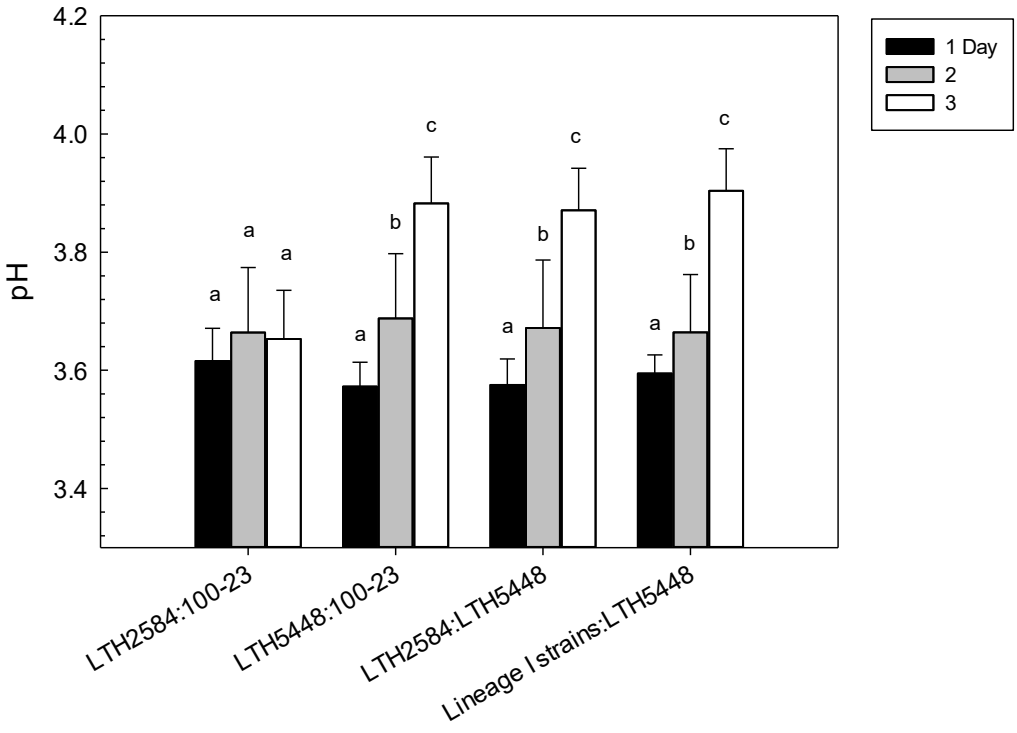

Figure 4-10 pH value of sourdoughs fermented with binary or quaternary strain combinations after 1, 2, or 3 days of fermentation

Data are shown as mean ± standard deviation of all 20 samples taken from each strain combination. Values that do not share a common superscript differ significantly ($p<0.05$)

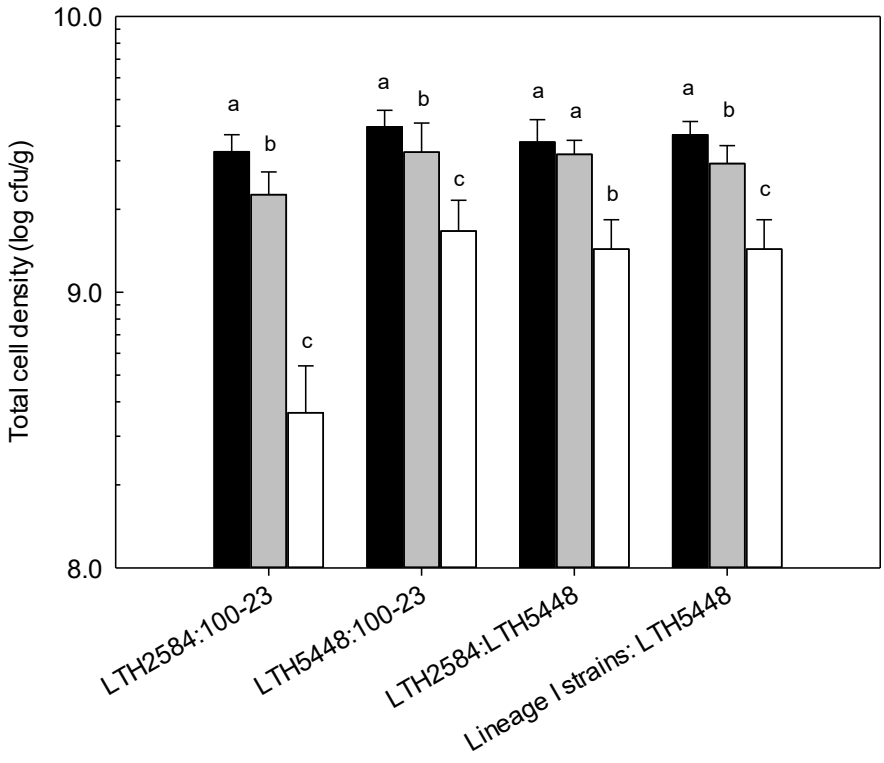

Figure 4-11 Total viable plate counts of sourdoughs fermented with binary or quaternary strain combinations

Total viable plate counts of sourdoughs fermented with binary or quaternary strain combinations after 1 day (**black bars**), 2 days (**gray bars**), or 3 days (**white bars**) of fermentation. Data are shown as mean ± standard deviation of all 20 samples taken from each strain combination. Values that do not share a common superscript differ significantly ($p<0.05$).

CHAPTER 5 Impact of probiotic *Lactobacillus* spp. on autochthonous lactobacilli in weaned piglets

5.1 Introduction

Weaning piglets undergo abrupt changes in diet, social and environmental conditions at weaning period, and are therefore susceptible to enteric pathogens including enterotoxigenic *Escherichia coli* and *Clostridium perfringens* (Gresse et al., 2017; Su et al., 2008). Feed antibiotics have been used to manage pig gut microbiota, however, these also contribute to development of antibiotic resistance (Wegener, 2003). Many jurisdictions restrict antibiotics to therapeutic use, therefore prohibiting the prophylactic use of antibiotics and antimicrobial growth promotors. Probiotic bacteria are an alternative to prophylactic antibiotics to prevent diarrheal disease in swine (Valeriano et al., 2017; Yang et al., 2015a; Le et al., 2016). Probiotic efficacy of *Lactobacillus* spp. to animal health and well-being has been extensively documented (for review, see Valeriano et al., 2017).

Selection criteria for identification of probiotic lactobacilli are currently lacking. It remains unclear whether health-promoting activities of lactobacilli are strain- or species-specific, or whether the characteristic is generally shared among *Lactobacillus* spp. Moreover, the genus *Lactobacillus* has an exceptional phylogenetic and physiological diversity. The genus encompasses 24 taxonomic groups; each of these represents a diversity that is typically observed in a bacterial genus (Zheng et al., 2015a). Lifestyles of *Lactobacillus* spp. were distinguished as

'free-living', 'nomadic' and 'host-adapted' (Duar et al., 2017b), based on the increasing availability of large-scale analysis of individual Lactobacillus species by large-scale competitive genomics in combination with ecological studies (Duar et al., 2017a and 2017b). Host adapted lactobacilli have a stable association with one or more species of vertebrate or insect hosts; free-living lactobacilli are adapted to environmental or plant-associated habitats; nomadic lactobacilli combined a free living lifestyle with the ability to temporarily persist in diverse animal or insect hosts (Duar et al., 2017b). This concept may provide a rational for selection of probiotics and an ecological perspective to interpret observations in human and animal trials. Host-adapted *Lactobacillus* spp. are present in high cell counts throughout the GI tract of pigs soon after birth and colonize the gastric epithelium (McGillivery and Cranwell, 1992; Tannock et al., 1987; Tannock and Smith, 1970). Members of the *Lactobacillus reuteri*-, *Lactobacillus delbrueckii*- and *Lactobacillus salivarius*-groups are dominant; the species *L. reuteri* and *Lactobacillus amylovorus* are most frequently isolated (Leser et al., 2002; Su et al., 2008; Valeriano et al., 2017). *L. reuteri* is further differentiated in host-adapted lineage that colonize the intestine of swine, chicken, rodents, and humans (Duar et al., 2017a; Su et al., 2012; Wegmann et al., 2015).

This chapter aims to determine whether host-adapted lactobacilli exhibit superior survival during gastrointestinal transit relative to nomadic and free-living organisms, and to characterize the impact of probiotic lactobacilli on autochthonous lactobacilli. This chapter employed *L. reuteri*, *L. casei*, a species with nomadic lifestyle without niche specialization (Broadbent et al., 2012;

Duar et al., 2017b) and *L. fermentum*, a species with a free-living lifestyle associated with plant material or environmental habitats (Duar et al., 2017b). The comparison of a reutericyclin producing strains of *L. reuteri* and its isogenic reutericyclin derivative (Lin et al., 2015; Gänzle, 2004) was used to assess the impact of specific antimicrobial metabolites on autochthonous lactobacilli (Yang et al., 2015b).

Vegetative cells of probiotic cultures are generally freeze-dried for use in food/ feed applications (Ross et al., 2005) but can be alternatively applied in feed fermentations (Le et al., 2016; Yang et al., 2015a). This study therefore delivered probiotic cultures as freeze-dried preparations or as fermentation organisms in fermented feed.

5.2 Materials and methods

5.2.1 Microorganisms and growth conditions

The reutericyclin producing *L. reuteri* TMW1.656 and the reutericyclin negative mutant *L. reuteri* TMW1.656Δ*rtcN* (Lin et al., 2015) and two commercial probiotics, *L. casei* K9-1 and *L. fermentum* K9-2 were routinely grown on MRS5 agar (Meroth et al., 2003) at 37°C at anaerobic conditions. Culture stocks were streaked onto MRS5 agar prior to overnight subculture in MRS5 broth at 37°C. *L. reuteri* TMW1.656 is a rodent-lineage representative of the vertebrate-host adapted species *L. reuteri* with documented probiotic activity in swine (Yang et al., 2015a); *L. casei* has been attributed a nomadic lifestyle and *L. fermentum* is associated with environmental habitats (Duar et al., 2017b). Food grade freeze-dried cultures of *L. casei* K9-1 and *L. fermentum*

K9-2 with a viable cell count of 10^9 CFU/g were provided by CanBiocin Inc. (Edmonton, AB, Canada). The freeze-dried cultures were stored at 4ºC until use.

5.2.2 Experimental diet preparation

Feed fermentations were performed as described (Yang et al., 2015a; Le et al., 2016). To confirm the identity of the inoculum with fermentation microbiota, the pH, the viable cell counts and the colony morphology of isolates from of each batch of fermented feed were monitored. Viable cell counts were determined by surface plating of serially diluted samples onto MRS5 agar.

The Phase I and II basal diets were fed sequentially in the 3-week pig trial and met recommended nutrient requirements for weaning piglets (Table 5-1 below). The Phase I basal diet was fed from day 0 to day 6, and the Phase II basal diet in the period from day 7 to 21 (Figure 5-7 below). The basal diet was mixed with wheat, fermented wheat or probiotic cultures to obtain the following dietary treatments: **Diet A**, unfermented wheat; **Diet B**, unfermented wheat artificially acidified with lactic acid and acetic acid (pH 3.8); **Diet C**, unfermented wheat mixed with freeze-dried culture of *L. casei* K9-1 and *L. fermentum* K9-2; **Diet D**, fermented wheat with *L. casei* K9-1 and *L. fermentum* K9-2; **Diet E**, fermented wheat with *L. reuteri* TMW1.656; **Diet F**, fermented wheat with isogenic mutant *L. reuteri* TMW1.656Δ*rtcN*. Cell counts of the probiotic strains *L. casei* K9-1, *L. fermentum* K9-2, *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN* in feed are shown in Table 5-2 below. The strains *L. casei* K9-1 and *L. fermentum* K9-2 were supplied as fresh culture in fermented feed, or as freeze-dried cultures. The average cell counts of *L. casei* K9-1 supplied in

Diet C and D were log 7.46 and 8.08 (CFU/g), respectively; the cell counts of *L. fermentum* K9-2 supplied in Diet C and D were log 7.25 and 7.68 (CFU/g), respectively. The estimated daily intake of individual probiotic strains was about 10^{10} -10^{11} CFU/day.

Table 5-1 Ingredients of basal diets

	Composition (%)	
Ingredient[a]	Phase I[b]	Phase II[b]
Wheat, hard red spring	20.00	50.00
Corn	31.54	1.76
Lactose	15.00	10.00
Soybean meal	15.00	15.00
Brassica napus canola meal		2.50
Soy protein concentrate	3.00	2.50
Herring meal	6.00	2.50
Corn DDGS[c]		5.00
Canola oil	4.00	3.40
Limestone	1.15	1.10
Salt	0.50	0.50
Other vitamin and mineral ingredients	3.31	5.24
TiO_2	0.50	0.50

[a]Composition of basal diets fulfills the National Research Council (NRC) recommendations (2012) for pigs (5-11 kg body weight).
[b]Phase I was day 0 to 6 and Phase II was day 7 to 21.
[c]DDGS, distillers dried grains with solubles.

5.2.3 Animal experiment

This study was performed at the University of Alberta Swine Research and Technology Centre (SRTC), University of Alberta (Edmonton, AB, Canada), was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care. A total of 48 crossbred castrated male piglets (Duroc × Large White/ Landrace F1) were selected at weaning (21 days old). Each piglet was housed in an individual metabolism pen (0.58 m width × 1.22 m length × 0.76 m height) in a temperature-

controlled room (28 ± 2.5ºC). The six dietary treatments were randomly allocated to 48 piglets in a randomized block design. Each pig was housed in a single pen to provide 8 replicates per dietary treatment. The six experimental diets were administered for 21 days and pigs were sacrificed on day 23. Pigs had free access to feed and water during the trial. Diets were provided twice a day according to the estimated daily feed consumption by the weaning piglets.

For bacterial analysis, fresh fecal grabs from the pen floor was collected from individual piglets at weaning day and days 7, 14 and 21. The fecal samples were kept at -20ºC immediately after arriving at laboratory. Digesta of stomach, ileum, cecum and colon was collected at euthanasia and stored at -20ºC freezer for subsequent analysis. Frozen samples were thawed, mixed aseptically by spatula and 2-3 g subsamples were stored at -80ºC.

5.2.4 Extraction of microbiota DNA from intestinal and fecal samples

DNA was extracted from intestine and fecal samples using QIAamp Fast DNA stool mini kit (Qiagen, Inc., Valencia, CA, USA). In brief, approximately 0.2 g of digesta or fecal sample into an autoclaved 2 mL tube filled with 0.2 g of silica beads. The weight of samples was recorded for subsequent calculation. The sample was subjected to mechanical homogenization followed by heating for 15min at 95ºC. The samples were processed according to the manufacturer's instructions; DNA extraction from pure cultures was performed using Blood & Tissue Kit (Qiagen, Inc., Valencia, CA, USA). The concentration of DNA was measured spectroscopically

(Nano-Drop, Thermo Fisher Scientific Inc., Wilmington, USA); and the quality of DNA extracted was evaluated according to the ratio of 260nm/280nm.

5.2.5 Establishment of strain-specific primers[15]

Strain-specific primers for *L. casei* K9-1 and *L. fermentum* K9-2 were identified by comparative genomic analysis with other strains of the same species. Genome sequencing, assembly, and annotation of probiotic strains *L. casei* K9-1 and *L. fermentum* K9-2 was conducted by Fusion Genomics (Burnaby, BC, Canada) using *L. casei* ATCC 393 and *L. fermentum* 3872 as reference genomes, respectively. Genome sequences for *L. casei* K9-1 and *L. fermentum* K9-2 were compared to the thirty-three sequenced genomes of *L. casei* and nineteen sequenced genomes of *L. fermentum*, respectively, that were available on May 15, 2016. Genome alignment for identification of unique sequences in the two strains was performed in MAUVE (Darling et al., 2004) (Figure 5-1 below, and Table 5-7 below).

Strain specific primers for *L. reuteri* strains targeted the reutericyclin biosynthesis gene cluster. This genomic island is exclusive to five strains of *L. reuteri* including *L. reuteri* TMW1.656 but is virtually absent from all other lactobacilli (Lin et al., 2015; Zheng et al., 2015b). Therefore, the non-ribosomal peptide synthase *rtcN* involved in reutericyclin production was used as DNA marker for *L. reuteri* TMW1.656 (Genbank accession number KJ659887.1). The mutant strain *L.*

[15] Contribution by Weilan Wang, Alysson Blaine, and Sarah Miyata-Kane

reuteri TMW1.656Δ*rtcN* was specifically detected with forward and reverse primer targets sites surrounding the deleted region of *rtcN*. The primer binding sites are also present in the wild type strain, however, elongation times prevented amplification of the 3047 bp *rtcN* from the wild type *L. reuteri* TMW1.656. Primers were synthesized at Integrated DNA Technologies (Coralville, IA, USA).

To evaluate primer specificity *in silico*, the designed primers were subjected to BLAST against the nucleotide collection available on Genbank in September 5, 2016 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In PCR reaction strain-specific primers resulted in positive amplicons from genomic DNA of respective probiotic strains (Table 5-8 below and Figure 5-6 below).

Table 5-2 Viable cell counts of probiotic strains in pig diets

	Cell count in diet (log CFU/g) [a,b,c]				
	L. casei K9-1	*L. fermentum* K9-2	*L. reuteri* TMW1.656	*L. reuteri* TMW1.656△*rtcN*	Total
Control	n.d.[c]	n.d.	n.d.	n.d.	n.d.
Acidified control	n.d.	n.d.	n.d.	n.d.	n.d.
Freeze-dried *L. casei*/*L. fermentum*	7.5±0.4	7.3±0.5	n.d.	n.d.	7.7±0.4
Fermented *L. casei*/*L. fermentum*	8.1±0.5	7.7±0.4	n.d.	n.d.	8.2±0.5
Fermented *L. reuteri* TMW1.656	n.d.	n.d.	8.4±0.5	n.d.	8.4±0.5
Fermented *L. reuteri* TMW1.656△*rtcN*	n.d.	n.d.	n.d.	8.3±0.5	8.3±0.5

[a] Cell counts of respective strains.
[b] Data is represented as mean ± SD. The average was calculated on 25 samples for each diet collected daily over the 3-week animal trial.
[c] n.d., cell counts were below the detection limit of 10^5 CFU/g.

Figure 5-1 Genome alignment of *L. casei* K9-1 and *L. fermentum* K9-2 against reference genomes[16]

(**A**) Genome alignment of *L. casei* K9-1 against genomes of *L. casei*. Shown is the comparison of an area of interest to the three of 33 strains that are most closely related to *L. casei* K9-1. The white area was selected as unique sequence region (highlighted by red box) for strain specific primer design. (**B**) Genome alignment of *L. fermentum* K9-2 against genomes of *L. fermentum*. Shown is the comparison of an area of interest to the two of 19 strains that are most closely related to *L. fermentum* K9-2. The brown block was selected as unique sequence region (highlighted by red box) for strain specific primer design.

5.2.6 *In silico* validation of group specific primers

Group-specific primers for *L. reuteri* group, *L. delbrueckii* group, *L. salivarius* group are shown in Table 5-3 below. The specificity of group-specific primers was verified with the probe match

[16] Contribution by Weilan Wang

tool of the Ribosomal Database Project (https://rdp.cme.msu.edu/). *L. reuteri* group specific primers target 11 out of 14 species in the *L. reuteri* group; *L. delbrueckii* group specific primers target 29 out of 36 species in the *L. delbrueckii* group; *L. salivarius* group specific primers target 14 out of 28 species in the *L. salivarius* group. Subsequently, group-specific primers were tested in PCR reactions with template DNA as follows: *L. plantarum* FUA3099, *L. fermentum* K9-2, *L. paralimentarius* FUA3121, *L. sanfranciscensis* FUA3458 and *L. casei* K9-1 were used for validation of the 'general LAB' primers; *L. ruminis* FUA3179, *L. animalis* FUA3045 were used for validation of '*L. salivarius* group' primers; *L. reuteri* TMW1.656 and *L. vaginalis* FUA3049 were used for validation of '*L. reuteri'* group primers; *L. crispatus* DSM29598 was used for validation of '*L. delbrueckii* group' primers.

Table 5-3 Primers used in PCR amplification[17]

Target	Primer	Sequence (5'-3')	Product size (bp)	Tm (°C)	Reference
Lactobacillus complex[a]	Lab F/ R	AGCAGTAGGGAATCTTCCA / CACCGCTACACATGGAG	341	63	(Walter et al., 2001)
L. reuteri group	sg-Lreu F/ R	GAACGCAYTGGCCCAA / TCCATTGTGGCCGATCAGT	289	60	(Matsuda et al., 2009)
L. delbrueckii group	sg-Ldel F/R	GATGCATAGCCGAGTTGAGAGACTGAT / TAAAGGCCAGTTACTACCTCTATCC	197	60	(Matsuda et al., 2009)
L. salivarius group	sg-Lsal F/R	CACCGAATGCTTGCAYTCACC / GCCGCGGGTCCATCCAAAA	182	60	(Matsuda et al., 2009)
L. casei K9-1	K9-1F/R	GTTGGAGGATCGCGGATTAG / CGTCACCGGAAGTGATGTT	98	62	This study
L. fermentum K9-2	K9-2F/R	CCCACGAGATTGCCCATATT / GAAGATCCATTGCCGTTTCATTAG	111	62	This study
L. reuteri TMW1.656	WT F/R	ACCGGAACATAACAACACCTTA / GAGGTTCCACCGTCATCAAA	105	62	This study
L. reuteri TMW1.656 △*rtcN*	rtcN F/R	ACGTTCTAGTAACACAAGTTGGA / TGTAGAGTGTGCTTGAGGAAAG	134	62	This study

[a] Lactic acid bacteria detected by these primers include *Lactobacillus* spp., *Pediococcus* spp., *Weissella* spp., and *Leuconostoc* spp.

[17] Partial contribution by Weilan Wang, Alysson Blaine, and Sarah Miyata-Kane

5.2.7 Quantitative PCR for detection of probiotic strains

The probiotic strains in intestinal and fecal samples were detected on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Standard curves for qPCR were generated with PCR amplicons obtained with the same primers and genomic DNA of the respective strains as template. Six 10-fold serially diluted standard samples were used as the template. The number of gene copies for each standard was calculated based on DNA concentrations as determined using Nano-drop spectrophotometer system (Thermo Fisher Scientific Inc., Wilmington, USA) and the molecular weight of the PCR product. The detection limit of the assay was 10^5 gene copies per gram. The qPCR reaction mixture with a total volume of 25 µL contains 12.5 µL of Quanti Fast SYBR Green master mix (Applied Biosystems), 0.5 µL of 10 µM forward/ reverse primer solution, 1 µL of template DNA solution and 10.5 µL of RNase-free water. qPCR amplification program was operated as follows, pre-denaturation stage at 95°C for 5 min; 40 cycles of three steps: denature at 95°C for 30 s, annealing stage at variable temperatures for 30 s, elongation at 72°C for 30 s; followed by melting stage with default settings. Technical repeats were conducted for all qPCR reactions.

5.2.8 High-resolution melting (HRM)-qPCR for detection of *Lactobacillus* groups

HRM-qPCR was conducted on Rotor-Gene Q (Qiagen, USA) using Type-it HRM PCR Kit (Qiagen, USA) (Lin and Gänzle, 2014a) with group specific primers listed in Table 5-3 above. Purified 16S rDNA amplicon derived from *L. ruminis* FUA3179, *L. reuteri* TMW1.656 and

L. crispatus DSM29598 were used as standards in quantification of *L. salivarius* group, *L. reuteri* group, *L. delbrueckii* group, respectively (Lin and Gänzle, 2014a). Multiple melting peaks shown in a melt-curve spectrum derived from the group-specific HRM-qPCR demonstrate multiple species exist within a given lactobacilli group. Multiple species were identified by Sanger sequencing of cloned PCR product. The cloning procedure was described as follows. In brief, 16S rDNA region was amplified from intestinal digesta DNA with group-specific primers followed by PCR product purification. The resultant purified 16S rDNA amplicon and the vector pUC19 isolated from *E. coli* FUA1293 were ligated by T4 ligase after simultaneously treated with FastDigest restriction enzyme SmaI. Subsequently, the ligation plasmid was transformed to competent cells of *E.coli* DH5α followed by plating onto LB agar containing IPTG (0.2 mM), X-gal (40 µg/mL) and Ampicillin (50 µg/mL). White colonies grown for 1 day were picked and then inoculated into LB broth. Plasmid was extracted from cell pellets for Sanger sequencing.

5.2.9 High throughput sequencing of 16S rRNA sequencing tags [18]

Exacted DNA was also used for 16S rRNA sequence analysis on an Illumina MiSeq performed by the University of Minnesota Genomics Center (Minneapolis, MN, USA). The V5-V6 domain of 16S rRNA gene was amplified using forward and reverse primers GTGCCAGCMGCCGCGGTAA and CGACRRCCATGCANCACCT, respectively, and the

[18] Contribution by Weilan Wang

amplicons were pooled for pair-end 300-bp reads sequencing. Sequences were analyzed on QIIME pipeline (MacQIIME 1.9.1-20150604) (Caporaso et al., 2010). After quality control, a total of 6,647,893 sequences with an average length of 266 bp were obtained, corresponding to an average 34,805 sequences per sample. Operational Taxonomic Units (OTU) clustering was conducted by UCLUST (Edgar, 2010) using GreenGenes database (released in May of 2013) with 97% similarity threshold after quality-filtering and de-multiplexing. Low abundance OTUs with relative abundance < 0.005% of the total OTUs were discarded (Bokulich et al., 2012). The OTU table was filtered by filter_taxa_from otu_table.py to obtain the OTUs clustered into *Lactobacillus*. The assignment of selected OTUs to phylogenetic groups in the genus *Lactobacillus* (Zheng et al., 2015a) was based on BLAST analysis with the sequences in the NCBI database. The relative abundance was calculated as percentage of the abundance of amplicons representing specific bacterial taxa relative to the total abundance of bacterial rDNA. Mixed Procedure (ProcMIXED) based on repeated measurement under randomized block design was applied to normalized relative abundance of each *Lactobacillus* groups.

5.2.10 Statistical analysis

Data analysis of qPCR results was performed in SigmaPlot (Systat Software, San Jose, CA). Gene copy numbers of administered probiotic strains, autochthonous *L. reuteri* group, *L. delbrucekii* group and *L. salivarius* group in intestinal contents were compared among dietary treatment using

two-way ANOVA. Statistical significance was assessed at an error probability of 5% ($P < 0.05$). Results were expressed as mean ± standard deviations.

5.3 Results

5.3.1 Strain-specific primers

A strain-specific quantitative PCR assay was established for the present study to monitor the fate of ingested probiotic strains during intestinal transit. Strain-specific primers for *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN* target the reutericyclin biosynthesis gene cluster which is unique to 5 strains of *L. reuteri* (Yang et al., 2015b; Lin et al., 2015). The strain-specific primers for probiotics *L. casei* K9-1 and *L. fermentum* K9-2 target unique sequences that were identified by comparative genomic analysis. Genomes of *L. casei* and *L. fermentum* were aligned and visualized using MAUVE (Figure 5-1 above) and the specificity of primers was verified by nucleotide BLAST on the NCBI database. Probiotic strains were detected in intestinal digesta and fecal samples from pigs receiving the corresponding strains, but not in pigs that were fed other probiotic strains, or pigs that did not receive probiotics (above). The detection limit of the qPCR assay for *L. casei* K9-1, *L. fermentum* K9-2, *L. reuteri* TMW1.656 and its mutant was 10^5 gene copies/g of wet feces or digesta (above). The unusual presence of probiotic strains in few fecal samples of probiotic-free groups (above) likely relates to cross-contamination during sampling or DNA handling. The unexpected presence of probiotic strain was not observed in intestinal samples excluding cross-contamination during feed preparation.

Table 5-4 Gene copy number of the orally administered probiotic strains in fecal samples

Microorganism and time (day)[a]	Log (gene copy#/g of wet feces) for the following diets[b]					
	Control	Acidified control	*L. casei*/*L. fermentum* freeze-dried	*L. casei*/*L. fermentum* fermented	*L. reuteri* TMW1.656 fermented	*L. reuteri* TMW1.656 △*rtcN* fermented
Lactobacillus complex						
0	9.2±0.9	9.2±0.7	9.4±0.9	9.8±0.4	9.7±0.9	9.3±0.8
7	10.0±0.3	10.3±0.6	10.3±0.6	10.5±0.7	9.9±0.5	9.7±0.5
14	10.0±0.6	9.9±0.8	10.3±0.3	10.0±0.7	9.6±0.6	10.0±0.7
21	9.7±0.8	9.6±0.5	9.7±0.6	10.3±0.7	9.6±0.6	9.5±0.5
L. reuteri group[c]						
0	8.7±1.4	8.1±1.2	8.8±1.8	9.2±1.2	8.6±1.6	9.4±1.4
7	10.1±0.5	10.0±0.8	10.4±0.2	9.6±0.7	9.8±1.3	9.4±1.5
14	10.2±0.4	10.4±0.4	10.3±0.4	10.1±0.5	9.8±0.6	9.7±0.8
21	10.2±0.5	9.9±0.4	10.0±0.3	10.3±0.5	9.5±0.5	9.6±0.7
L. casei K9-1						
0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
7	<5.0	<5.0	8.8 ± 0.3^{B}	8.5 ± 0.5^{B}	<5.0	<5.0
14	<5.0	<5.0	9.4 ± 0.7^{A}	8.7 ± 0.7^{B}	<5.0	<5.0
21	<5.0	<5.0	9.1 ± 0.4^{A}	8.8 ± 0.3^{A}	<5.0	<5.0
L. fermentum K9-2						
0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
7	<5.0	<5.0	5.9 ± 0.7^{D}	6.8 ± 1.1^{C}	<5.0	<5.0
14	<5.0	<5.0	6.4 ± 0.6^{C}	6.7 ± 0.5^{C}	5.6±0.6	<5.0
21	<5.0	<5.0	6.0 ± 0.8^{B}	6.8 ± 0.5^{B}	<5.0	<5.0
L. reuteri TMW1.656						
0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0
7	<6.0	<6.0	<6.0	<6.0	9.8 ± 0.7^{A}	<6.0
14	<6.0	<6.0	<6.0	<6.0	9.4 ± 0.4^{A}	<6.0
21	<6.0	<6.0	<6.0	<6.0	9.3 ± 0.2^{A}	<6.0
L. reuteri TMW1.656△*rtcN*						
0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0
7	<6.0	<6.0	<6.0	7.2±0.8	<6.0	9.2 ± 0.5^{AB}
14	<6.0	<6.0	<6.0	<6.0	<6.0	8.9 ± 0.7^{AB}
21	<6.0	<6.0	<6.0	<6.0	<6.0	8.9 ± 0.3^{A}

[a]Feces were collected weekly in the 3-week experiment, at day 0, 7, 14 and 21.

[b]Gene copy number of respective probiotic strains was determined by qPCR assay. Probiotic strains added to respective were indicated in method section. Data are presented

as mean ± SD of 8 pigs. One-way ANOVA was applied to compare probiotic strains in gene copy number of the same collection day. Data without a common capital superscript in the same collection day differ ($P < 0.05$).
[c]*L. reuteri* group quantified in this assay consists of 11 individual species of *Lactobacillus* as validated by *in silico* analysis.

5.3.2 Fate of ingested probiotic strains through the piglet GIT

To assess survival and persistence of ingested probiotic strains with different evolutionary backgrounds, probiotic strains were quantified in digesta obtained from the stomach, the ileum, the caecum, and the colon (Figure 5-2 below) and in fecal samples (above). The bacterial load for administered probiotic strains gradually declined from the proximal to distal GIT of piglets (Figure 5-2 below); gene copy numbers in colonic digesta were significantly lower than gene copy numbers in stomach or illeal digesta for all strains except *L. reuteri* TMW1.656Δ*rtcN* ($P < 0.05$). The decline in the number of *L. fermentum* K9-2 from proximal GIT (stomach) to distal GIT (colon) was largest relative to other probiotic strains. Gene copy numbers of *L. fermentum* K9-2 in the cecum were lower ($P < 0.05$) when delivered as freeze-dried form compared to delivery of the same strain in fermented feed (Figure 5-2 below). *L. reuteri* wild type strain had higher gene copy number in stomach than its reutericyclin-negative isogenic mutant (Figure 5-2 below). Conforming to the abundance of strain specific DNA in intestinal samples, higher gene copies of the *L. reuteri* strains were detected in fecal samples when compared to *L. casei* K9-1 and *L. fermentum* K9-2 (above). Overall, the strain specific detection of probiotic lactobacilli in intestinal and fecal samples indicates that that the survival of vertebrate host-adapted lactobacilli is higher when compared to other lactobacilli.

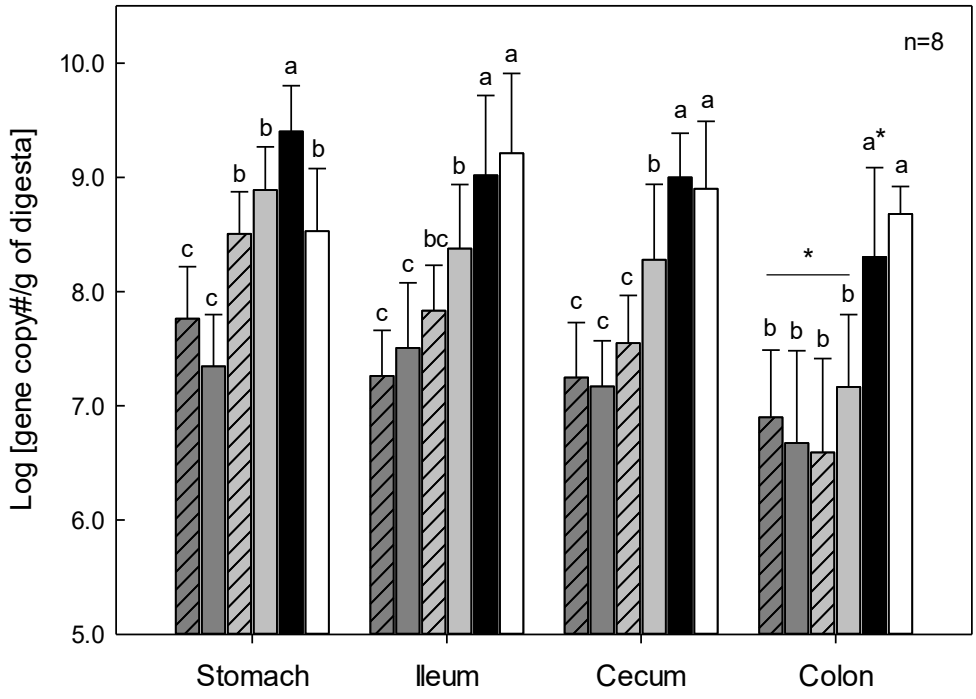

Figure 5-2 Quantification of probiotic lactobacilli in stomach, illeal, cecal, and colonic digesta

Animals received diets containing *L. casei* K9-1 (**dark gray bars**) and *L. fermentum* K9-2 (**light gray bars**) in freeze-dried form (**hatched bars,** Diet C), or through fermentation (Diet D), *L. reuteri* TMW1.656 (**black bars**, Diet E) or TMW1.656Δ*rtcN* (**white bars**, Diet F). Probiotic organisms were detected with strain specific primers to quantify *L. casei* K9-1 (**dark gray bars**); *L. fermentum* K9-2 (**light gray bars**); *L. reuteri* TMW1.656 (**black bars**), and *L. reuteri* TMW1.656Δ*rtcN* (**white bars**). Data are presented as mean (n=8) ± standard deviations of 8 replicate observations. One-way ANOVA was performed to assess differences of gene copy numbers between different strains in the same region of gut. Lack of common lower case letters means difference between two strains in same region of gut ($P < 0.05$). Gene copy numbers of strains in colonic digesta are marked with an asterisk if they were lower ($P < 0.05$) in comparison to gene copy numbers of the same strain in stomach digesta.

5.3.3 Effect of probiotic strains on quantity and composition of autochthonous lactobacilli

To characterize the impact of probiotic lactobacilli on autochthonous lactobacilli, the abundance and composition of autochthonous lactobacilli was analyzed in digesta samples collected at the end of the trial. Intestinal lactobacilli all belonged to the *L. reuteri* group, the *L. delbrueckii* group,

or the *L. salivarius* group (Table 5-5 below). Group-specific HRM-qPCR quantified all representatives of these *Lactobacillus* groups. HRM-qPCR additionally detects whether multiple species are present within each group if the melting temperatures of amplicons from different species differs. *L. reuteri* group constituted the most abundant group in the stomach; the *L. delbrueckii* group was most abundant in colonic digesta while the *L. salivarius* group was a minor component in both intestinal compartments (Figure 5-3 below). Probiotics did not alter the composition of autochthonous *Lactobacillus* populations in the stomach (Figure 5-3A below). Conversely, the abundance of the *L. salivarius* group in the colon was substantially decreased in animals receiving chemically acidified feed or fermented feed, indicating that organic acids may contribute to this modulation (Figure 5-3B below).

HRM-qPCR discriminates between 16S rDNA amplicons obtained with the same primers by analysis of the melting temperature, and thus discriminates between closely related species which differ with respect to the melting temperature (Tm) of the respective 16S rDNA amplicons. Melting peaks obtained in HRM-qPCR analysis were assigned to specific *Lactobacillus* species by using reference strains, Sanger sequencing of PCR amplicons, and 16S rRNA sequences from fecal samples of same piglet. Two melting peaks with Tm 81.8ºC and 82.9ºC were consistently observed after amplification of 16S rDNA from the *L. salivarius* group in colonic digesta of 10 piglets from all six dietary treatments. Only one melting peak with a Tm of 81.8ºC occurred for the remaining 38 piglets (Figure 5-4A below). Sanger sequencing of PCR amplicons from one of

the piglets identified amplicons with 81.8ºC and 82.9ºC as amplicons from *L. salivarius* and *L. ruminis*, respectively (Figure 5-4A below). This assignment matched the predicted Tm 16S rDNA from *L. ruminis* and *L. salivarius*. *L. salivarius* accounted for about 99% of *L. salivarius* groups organisms in fecal samples (Table 5-5 below, and data not shown), in agreement with the consistent presence of the amplicon with Tm 81.8ºC in intestinal samples from all piglets (Figure 5-4A below). In colonic digesta, a single melting peak at Tm 84.1ºC was observed with *L. reuteri* group specific primers (Figure 5-4B below). This Tm matches the Tm of the reference strain of *L. reuteri,* and the prevalence of OTUs assigned to *L. reuteri* in 16S sequences of fecal samples. A single peak at Tm of 84.5ºC was observed in all samples with primers specific for the *L. delbrueckii* group (Figure 5-4C below). This Tm matches the predicted Tm of *L. amylovorus* (84.75°C), *L. johnsonii* (85.0°C), as well as *L. gasseri* (84.75°C); these species can thus not be differentiated by the HRM-qPCR assay employed in this study. OTUs assigned to *L. amylovorus* or *L. gasseri* / *L. johnsonii* accounted for more than 97% of all sequences assigned to the *L. delbrueckii* group in fecal samples. HRM-qPCR of intestinal samples provided no indication that probiotic feeding influenced the composition of intestinal lactobacilli at the species level.

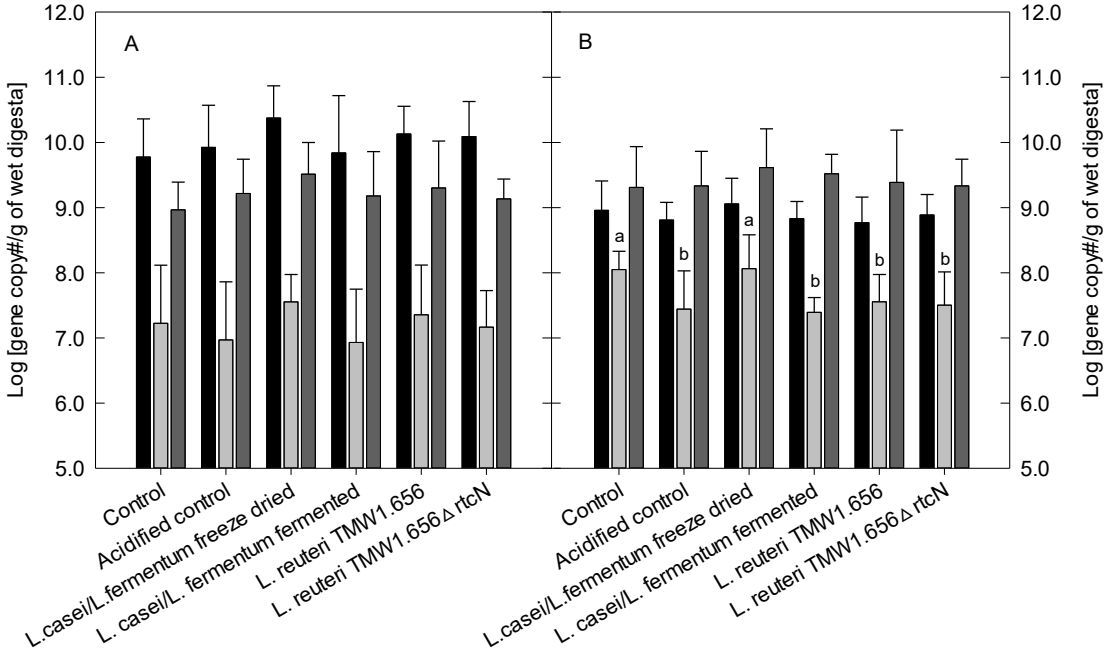

Figure 5-3 Gene copy numbers of autochthonous *Lactobacillus* groups in the stomach and the colon of piglets

Gene copy numbers of *L. reuteri* group (**black bar**), *L. salivarius* group (**gray bar**), *L. delbrueckii* group (**white bar**) in wet digesta obtained from the stomach (**Panel A**) and the colon (**Panel B**) of piglets. Data is expressed as mean ± SD of 8 pigs. The gene copy number was quantified using HRM-qPCR method with a detection limit of 1×10^5 gene copies/g of wet digesta. Significant differences between gene copy numbers from pigs fed different diets were assessed by one-way ANOVA. Bars without a common superscript differ ($P < 0.05$); superscripts are not shown where values were not different.

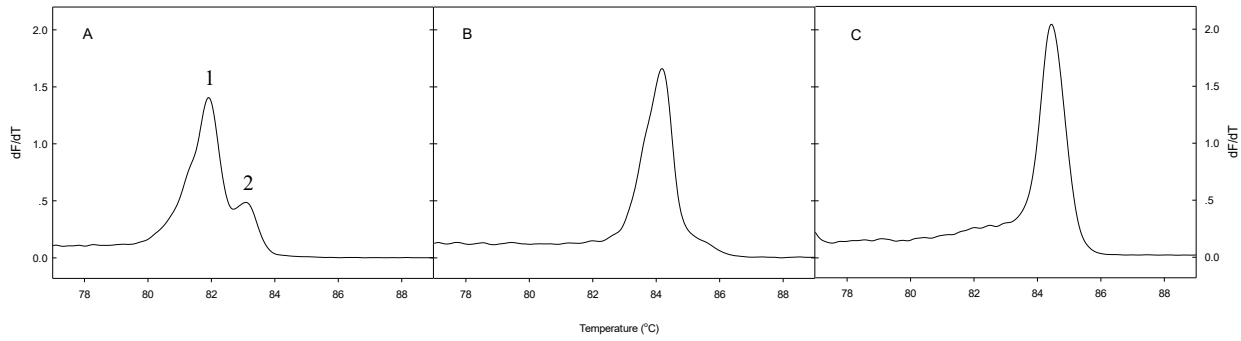

Figure 5-4 First derivatives of melt curves of PCR products obtained from digesta microbiota DNA with *Lactobacillus* group-specific primers

(**Panel A**) *L. salivarius* group primer. Based on the Sanger sequencing and Tm values of reference strains, two peaks shown in above spectrum are presumably assigned to *L. salivarius* (Peak 1 as marked on the curve) and *L. ruminis* (Peak 2 as marked on the curve). OTU's matching to *L. salivarius* was most abundant in 16S sequencing. Colon digesta of Piglet#10 was used for melt curves shown. (**Panel B**) *L. reuteri* group primer. The peak shown in above spectrum is assigned to *L. reuteri* matching the Tm value of the reference strain. OTU's matching to *L. reuteri* was most abundant in 16S sequencing. Colon digesta of Piglet#10 was used for melt curves shown. (**Panel C**) *L.delbrueckii* group primer. The peak shown in above spectrum was assigned to *L. amylovorus* or *L. johnsonii* or *L. gasseri* based on estimated Tm value of respective species and fecal OTU composition. Colon digesta of Piglet#10 was used for melt curves.

Table 5-5 Abundance of rDNA corresponding to the *L. reuteri* group, *L. salivarius* group, *L. delbrueckii* group and *Lactobacillus* spp. relative to total bacterial rDNA in feces of piglets during the first 3 weeks post weaning

Group / Collection day	Control	Acidified control	*L. casei* / *L. fermentum* freeze-dried	*L. casei* / *L. fermentum* fermented	*L. reuteri* TMW1.656 fermented	*L. reuteri* TMW1.656Δ*rtcN* fermented
L. reuteri group						
0	0.6±0.7 $^{B, b}$	2.1±3.2 ^{A}	1.7±2.0 $^{AB, b}$	0.8±1.0 $^{B, b}$	1.7±2.4 ^{AB}	0.6±0.8 $^{B, b}$
7	1.7±1.2 $^{AB, a}$	3.7±3.9 ^{AB}	2.5±1.6 $^{AB, ab}$	4.7±4.0 $^{A, a}$	1.6±1.3 ^{B}	2.1±2.3 $^{AB, ab}$
14	3.4±3.3 $^{A, a}$	2.7±1.8 ^{AB}	3.6±2.1 $^{A, a}$	2.3±1.2 $^{AB, a}$	1.1±0.5 ^{B}	2.1±1.7 $^{AB, a}$
21	1.6±0.6 $^{AB, a}$	2.5±2.4 ^{AB}	2.1±1.2 $^{AB, ab}$	2.7±1.4 $^{A, a}$	1.0±0.3 ^{B}	2.7±2.5 $^{AB, a}$
L. salivarius group						
0	0.05±0.06	0.10±0.16	0.04±0.06	0.03±0.07	0.08±0.12	0.02±0.04
7	0.03±0.05 ^{AB}	0.03±0.07 ^{AB}	0.08±0.11 ^{AB}	1.7±4.7 ^{A}	0.08±0.13 ^{AB}	0.01±0.02 ^{B}
14	0.02±0.02	0.02±0.03	0.03±0.07	0.03±0.04	0.01±0.01	0.01±0.02
21	0.05±0.10	0.03±0.07	0.01±0.01	0.04±0.05	0.00±0.00	0.01±0.01
L. delbrueckii group						
0	1.9±1.5 $^{B, b}$	2.9±3.3 $^{AB, b}$	3.0±2.6 $^{AB, b}$	1.9±1.7 $^{B, b}$	4.0±4.1^{A}	2.5±3.6$^{AB, b}$
7	6.8±6.5 ^{a}	11.5±7.3 ^{a}	7.4±4.8 ^{a}	12.6±13.7 ^{a}	6.2±4.6	9.1±12.1 ^{a}
14	4.6±2.9 ^{a}	5.6±4.6 ^{ab}	6.0±2.7 ^{a}	5.9±4.1 ^{a}	4.2±2.0	9.1±6.7 ^{a}
21	5.5±3.5 ^{a}	8.8±4.0 ^{a}	5.3±2.0 ^{a}	6.0±3.3 ^{a}	7.1±3.7	6.8±3.7 ^{a}
Lactobacillus spp.						
0	2.5±1.6 $^{B, b}$	5.1±5.8 $^{A, b}$	4.8±4.4 $^{A, b}$	2.7±2.5 $^{B, b}$	5.8±6.2 ^{A}	3.1±4.3$^{AB, b}$
7	8.5±7.5 $^{B, a}$	15.2±6.3 $^{A, a}$	10.0±5.0 $^{AB, a}$	19.1±20.5 $^{A, a}$	7.9±5.0 ^{B}	11.2±14.3$^{AB, a}$
14	8.0±3.5$^{AB, a}$	8.3±4.9 $^{AB, ab}$	9.6±3.7 $^{A, a}$	8.3±4.8 $^{AB, a}$	5.3±2.5 ^{B}	11.2±7.1$^{A, a}$
21	7.1±3.6 ^{a}	11.3±5.3 ^{a}	7.5±2.5 ^{a}	8.7±3.1 ^{a}	8.0±3.8	9.6±5.4 ^{a}

Note: Data were determined by sequencing of 16S rRNA tags, and are represented as mean ± SD of 8 pigs. Within a row, means without a common capital superscript differ ($P < 0.05$). Within a column, means without a common lowercase superscript differ ($P < 0.05$).

5.3.4 Effect of probiotic strains on autochthonous *Lactobacillus* communities in feces

The alteration of intestinal Lactobacillus communities in response to probiotics was further analyzed by high throughput sequencing of 16S rRNA sequence tags of PCR amplicons from fecal samples. In keeping with prior observations with *L. reuteri* TMW1.656 in weanling piglets (Yang et al., 2015b), probiotic feeding did not induce major change for total fecal microbiota (Figure 5-8 below). Sequences matching to the genus *Lactobacillus* were initially analyzed at the level of the phylogenetic group (Zheng et al., 2015). Identification of most lactobacilli at the species level was achieved by a combination of 16S rRNA sequence data and the species-level identification with HRM-qPCR (Table 5-5 above, and Figure 5-5 below). Individual animals differed substantially with respect to the *Lactobacillus* microbiota at baseline (Table 5-5 above). The abundances of overall lactobacilli and the three specific *Lactobacillus* groups fluctuated during the first two weeks while stabilized after Week 2 (Table 5-5 above, and Figure 5-5 below). Probiotic interventions with *L. casei* and *L. fermentum* did not affect the abundance of *L. reuteri* group, *L. delbrueckii* group and *L. salivarius* group (Table 5-5 above). Likewise, abundance and composition of intestinal lactobacilli were not affected by administration of probiotic strains in freeze-dried form or in fermented feed (Table 5-5 above). Administration of the reutericyclin positive *L. reuteri* TMW1.656 transiently decreased the abundance of the *L. reuteri* group in fecal samples in comparison to the control group and *L. casei / L. fermentum* fed animals (Table 5-5 above) and transiently reduced the proportion of total lactobacilli in comparison to the

reutericyclin-negative mutant (Figure 5-5 below). Taken together, these results suggest that reutericyclin is a subtle contributor to shaping the *Lactobacillus* community in pigs.

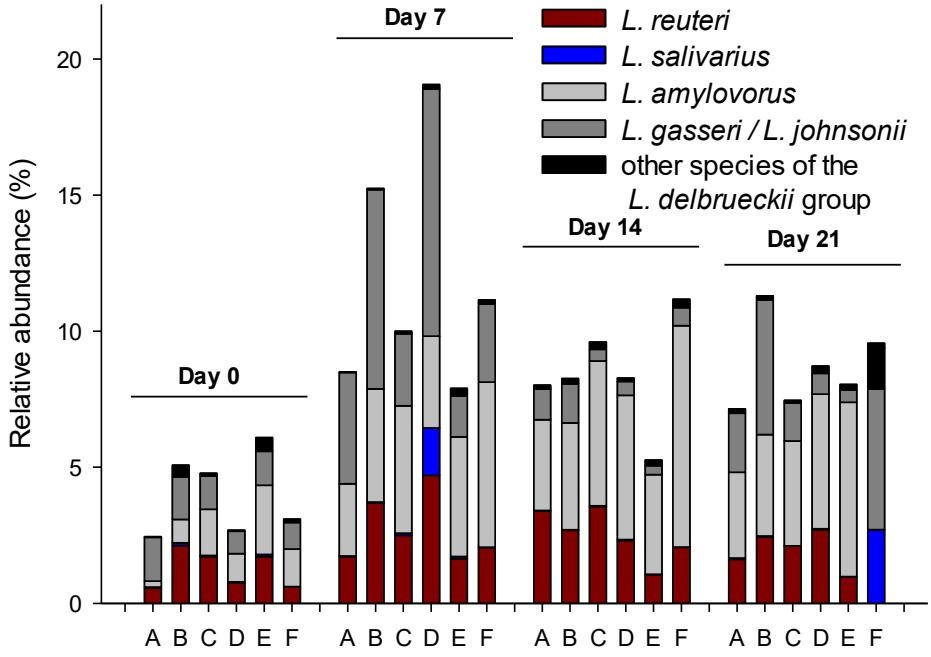

Figure 5-5 Relative abundance of the species *L. reuteri*, *L. salivarius*, *L. amylovorus*, *L. gasseri* / *L. johnsonii*, and other members of the *L. delbrueckii* group in feces of pigs during the first three weeks post weaning[19]

Sequencing of 16S rRNA tags allowed the assignment of *Lactobacillus* sequences at the level of the phylogenetic group; the assignment of sequences of specific *Lactobacillus* species was enabled by combination of rDNA sequence data with HRM-qPCR and Sanger sequencing of PCR amplicons. Bars indicate the average relative abundance of *Lactobacillus* spp. compared to the total fecal microbiota. Different colors represent different species as indicated; letters indicate the different diet as follows. Diet A: control, Diet B: acidified control, Diet C: *L. casei* K9-1 and *L. fermentum* K9-2 in freeze-dried form, Diet D: *L. casei* K9-1 and *L. fermentum* K9-2 in fermented form, Diet E: *L. reuteri* TWM1.656, Diet F: *L. reuteri* TMW1.656Δ*rtcN*. Data without a common capital superscript differ ($P < 0.05$).

5.4 Discussion

The present study investigated the interaction between ingested probiotic bacteria of non-swine origin and autochthonous *Lactobacillus* community in the pig intestine. We employed probiotic *L. fermentum*, *L. casei*, and *L. reuteri* strains on the weaned piglet model to i) compare effect of

[19] Contribution by Weilan Wang

freeze-dried culture versus fermented cultures on probiotic efficacy in piglet GIT; ii) develop a culture-independent quantitative method for ingested probiotic strains, and follow their fates during gastric transit; iii) assess *in vivo* ecological role of the reutericyclin producer in the swine intestine. In the present study the absolute amount and relative abundance of three autochthonous *Lactobacillus* groups in various gut regions (i.e. stomach, ileum, cecum and colon) and feces were detected using strain-, group-specific HRM-qPCR coupled with 16S amplicon sequencing.

5.4.1 Development of strain-specific qPCR assay

Strain-specific quantitative PCR differentiated probiotic strains from autochthonous lactobacilli throughout the intestine of weaned piglets. The availability of a large number of genomes of lactobacilli allows identification of strain specific DNA markers by *in silico* comparative genomics. Prior to the availability of genome sequence data for multiple strains of the same species, typing methods, such as Random Amplification of Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP) and Suppression Subtractive Hybridization (SSH) were used to distinguish between bacterial isolates (Ahlroos and Tynkkynen, 2009; Fujimoto et al., 2008; Maruo et al., 2006; Tilsala-Timisjärvi and Alatossava, 1998; Bunte et al., 2000). Studies employing these assays are summarized in Table 5-6 below. Limitations of these assays include the limited specificity and the requirement for bacterial culture. For example, RAPD and AFLP analyses require re-isolation of strains and cover only 1-10% of a bacterial genome, which may be insufficient for strain-level differentiation. SSH

relies on the comparison to only one closely related organism (Konstantinov et al., 2005; Sattler et al., 2014). The present study thus provides a novel approach for strain-specific quantification of probiotic *L. fermentum*, *L. casei* and *L. reuteri* by qPCR.

Table 5-6 Recent reports about strain-specific identification or quantification methods

Strain	Culture independent (Yes/No)	Method	Identification/ quantification	Sample type	Ref.
L. reuteri DSM 16350	Yes	SSH & strain specific qPCR	Quantification	Chicken feed and intestine	(Sattler et al., 2014)
L. sobrius 001	Yes	Representational difference analysis (RDA) & strain-specific qPCR	Quantification	Pure culture mix	(Konstantinov et al., 2005)
L. rhamnosus GG	Yes	RAPD & strain-specific qPCR	Quantification	Human feces	(Ahlroos and Tynkkynen, 2009)
Lactococcus lactis subsp. *cremoris* FC	Yes	RAPD & strain-specific qPCR	Quantification	Human feces	(Maruo et al., 2006)
B. longum Y10, *B. infantis* Y1 and *B. breve* Y8	Yes	ITS (*in silico* comparison) & strain-specific qPCR	Quantification	Yogurt/ Human feces	(Vitali et al., 2003)
L. gasseri 4B2	No	Colony-multiplex PCR	Identification	Mouse feces	(Lucchini et al., 1998)
L. rhamnosus Lc 1/3	Yes	RAPD & PCR	Identification	Pure culture mix	(Tilsala-Timisjärvi and Alatossava, 1998)
L. paracasei LTH 2579	No	Subtraction hybridization & PCR	Quantification	Fermented sausage/ human feces	(Bunte et al., 2000)
L. paracasei IMPC2.1	Yes	f-AFLP & PCR	Identification	Pure culture mix	(Sisto et al., 2009)
L. rhamnosus 35	Yes	Subtractive hybridization & PCR	Identification	Pure culture mix	(Coudeyras et al., 2008)
B. bifidum BF-1	Yes	RAPD-qPCR	Quantification	Human feces	(Fujimoto and

Table 5-6 Recent reports about strain-specific identification or quantification methods

Strain	Culture independent (Yes/No)	Method	Identification/ quantification	Sample type	Ref.
					Watanabe, 2013)
L. gasseri K7	Yes	qPCR targeting bacteriocin gene	Quantification	Human feces	(Treven et al., 2013)
L. casei strain Shirota	Yes	RAPD & strain-specific qPCR	Quantification	Human feces	(Fujimoto et al., 2008)
L. reuteri TMW1.656	Yes	*in silico* comparison & strain-specific qPCR targeting RTC biosynthesis gene	Quantification	Pig intestine and feces	(Yang et al., 2015a)
Bifidobacterium longum AH1206	Yes	*in silico* comparison & strain-specific qPCR	Quantification	Human feces	(Maldonado-Gómez et al., 2016)
L. salivarius abp118	No	Selective medium for rifampicin resistance plasmid	Quantification	Mouse and pig intestine and feces	(Riboulet-Bisson et al., 2012)
L. reuteri ATCC PTA 6475 and *L. mucosae* FSL-04	No	RAPD typing	Quantification	Human feces	(A. Frese et al., 2012)
Bifidobacterium breve Yakult (BbrY)	No	RAPD & strain-specific qPCR	Quantification	Human feces	(Fujimoto et al., 2011)

5.4.2 Survival of freeze-dried and fresh probiotic cultures

The survival of *L. fermentum* K9-2 in the GI tract was increased when this strain was as part of fermented feed compared to delivery of the same strain as freeze-dried culture. In contrast, the survival of *L. casei* K9-1 did not depend on the form of delivery. The survival of freeze-dried probiotic strains during intestinal transit is affected by multiple factors including the culture conditions and the pH at harvest of probiotic bacteria, the use of cryoprotectants during freeze drying, and the composition of the food matrix used for probiotic delivery (Vinderola et al., 2012). In feed applications, endospores of Bacillus spp. are currently preferred as probiotic additives due to the resistance of Bacillus endospores to the high temperatures during feed production and feed distribution (Krause et al., 2010; Zani et al., 1998). Feed fermentation is thus a viable alternative for strain-specific delivery of probiotics in animal production that eliminates the need for strain preparations with high resistance to heat and dry storage (Le et al., 2016). In analogy, the use of probiotic strains as starter cultures in food fermentations (Marco et al., 2017) may improve their survival during intestinal transit.

5.4.3 Lifestyles of lactobacilli relate to intestinal survival

Strain-specific primers provided a powerful tool to analyze survial of probiotic lactobacilli and their interaction with autochthonous lactobacilli. The lifestyle of lactobacilli was proposed to determine their suitability for probiotic applications (Duar et al., 2017b; Walter et al., 2018); however, this claim was not substantiated experimentally. *L. casei*, *L. fermentum* and *L. reuteri* used in the present study represent nomadic, free-living and host-adapted organisms, respectively (Duar et al., 2017b). The host-adapted *L. reuteri* strain exhibited superior persistence in the swine intestine compared to nomadic or free-living species (Figure 5-2 above, and above). Our study complements and expands observations in human subjects (Frese et al., 2012) *L. reuteri* is a

symbiont of pig, and pig isolates have evolved into a phylogenetic clade IV distinct from other host-specific isolates (Frese et al., 2011). However, genetic signatures distinguishing pig-derived *L. reuteri* from the isolates from other origins are lacking (Wegmann et al., 2015), and clade IV pig isolates do not outcompete other strains of *L. reuteri* in the pig gut (Duar et al., 2017a). The lack of swine-specific metabolic traits of L. reuteri may account for the survival of *L. reuteri* TMW1.65, a sourdough isolate of the rodent-specific clade III (Su et al., 2012; Zheng et al., 2015b) in the pig intestine. The present study thus supports the hypothesis that adaptation of lactobacilli to vertebrate hosts is a relevant criterion for selection of probiotic strains (Duar et al., 2017b; Walter et al., 2018).

5.4.4 Impact of probiotic strains on autochthonous lactobacilli

Despite colonization resistance of intestinal microbiota, increasing evidence indicates a role of probiotic strains on modulating autochthonous microbiota, if strains are adapted to vertebrate hosts, or to specifically to the host species. Generally, probiotics have only a limited impact on the resident gut microbiota (McNulty et al., 2011; Zhang et al., 2016), and probiotic strains are detectable only for a few days after intake of the probiotic administration ends (Derrien and van Hylckama Vlieg, 2015). However, temporary or permanent persistence of host-adapted probiotic strains was observed when the ecological niche was not occupied by closely related species and when the probiotic strain was adapted to the host species (Maldonado-Gómez et al., 2016; Tannock et al., 2000). The present studies investigated weaned piglets, which undergo major shifts in intestinal microbiota including intestinal lactobacilli in the first two weeks post weaning (Janczyk et al., 2007; Pieper et al., 2006; Su et al., 2008; Yang et al., 2015b; Le et al., 2016) (Figure 5-5 above). Feed fermentation and probiotic lactobacilli were subtle yet significant modulators on the population of autochthonous lactobacilli. The abundance of the *L. salivarius*

group in colonic samples was decreased by feed that was acidified chemically or by fermentation (Figure 5-3 above). Strain-specific effects of probiotics on composition of intestinal lactobacilli were observed only for the reutericyclin producing *L. reuteri* TMW1.656. Production of antimicrobial metabolites by probiotic strains is regarded as an important trait for probiotic functionality; past studies particularly discussed bacteriocin formation as a potential probiotic trait (Dobson et al., 2012; Riboulet-Bisson et al., 2012). *L. reuteri* TMW1.656, a strain producing low-molecular weight antimicrobial compound reutericyclin, affected intestinal microbiota of piglets when compared to a reutericyclin-negative wild type strain ($P < 0.05$) (Yang et al., 2015b). We investigated the role of reutericyclin in shaping Lactobacillus populations, by comparison of *L. reuteri* TMW1.656 to a reutericyclin-deficient isogenic mutant (Figure 5-2 above). The reutericyclin producing L. reuteri TMW1.656 persisted better in the stomach of piglets when compared to the reutericyclin-negative mutant. The reutericyclin production by *L. reuteri* TMW1.656 also altered fecal lactobacillus communities (Table 5-5 above and Figure 5-2 above), indicating that reutericyclin production may displace sensitive autochthonous lactobacilli.

In conclusion, the present compared the survival of lactobacilli with different lifestyles in the swine intestinal tract. *L. reuteri*, a species adapted to vertebrate hosts, survives better during intestinal transit of weaned piglets compared to either the nomadic *L. casei* or to the free-living *L. fermentum*. Therefore, ecology and lifestyle of *Lactobacillus* strains may be suitable criteria to select probiotic strains for use in swine production. Probiotic lactobacilli had only a limited impact on autochthonous lactobacilli in the swine intestine, however, reutericyclin production has a subtle but significant impact on intestinal microbiota. Probiotic lactobacilli that were delivered with feed fermentations persisted equal to or better in the swine intestine when compared to freeze-dried

cultures, indicating that feed fermentation with probiotic cultures is an alternative to freeze-dried cultures or bacterial endospores.

5.5 References

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5.6 Supplementary materials

5.6.1 Multiple genome alignment and strain specific primer design[20]

5.6.1.1 Multiple genome alignment and unique sequence selection

The principle of strain specific primer design was based on the unique nucleotides searching of the target strain compared to other strains from the same species. Multiple genome alignments were conducted to seek these unique sequences in genomes of *L. casei* K9-1 and *L. fermentum* K9-2. As listed in Table 5-7 below, 33 genomes of *L. casei* and 19 genomes of *L. fermentum* were downloaded from National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) FTP site and applied as reference genomes. Progressive Mauve algorithm (The Darling lab at the University of Technology Sydney, Australia) was used for the comparative sequence analysis. The alignment of multiple genomes was described in detailed in method, result sections of main text.

Table 5-7 Genomes used for multiple genome alignment

Genome Accession	NCBI FTP site
Lactobacillus casei	
GCA_000014525.1_ASM1452v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/014/525/GCA_000014525.1_ASM1452v1
GCA_000019245.3_ASM1924v3	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/019/245/GCA_000019245.3_ASM1924v3
GCA_000026485.1_ASM2648v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/026/485/GCA_000026485.1_ASM2648v1
GCA_000194765.1_ASM19476v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/194/765/GCA_000194765.1_ASM19476v1
GCA_000194785.1_ASM19478v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/194/785/GCA_000194785.1_ASM19478v1
GCA_000309565.2_ASM30956v2	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/565/GCA_000309565.2_ASM30956v2
GCA_000309585.1_ASM30958v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/585/GCA_000309585.1_ASM30958v1
GCA_000309605.1_ASM30960v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/605/GCA_000309605.1_ASM30960v1
GCA_000309625.1_ASM30962v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/625/GCA_000309625.1_ASM30962v1
GCA_000309645.1_ASM30964v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/645/GCA_000309645.1_ASM30964v1

[20] Contribution by Weilan Wang

Table 5-7 Genomes used for multiple genome alignment

Genome Accession	NCBI FTP site
GCA_000309665.1_ASM30966v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/665/GCA_000309665.1_ASM30966v1
GCA_000309685.1_ASM30968v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/685.GCA_000309685.1_ASM30968v1
GCA_000309705.1_ASM30970v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/705/GCA_000309705.1_ASM30970v1
GCA_000309725.1_ASM30972v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/725/GCA_000309725.1_ASM30972v1
GCA_000309745.1_ASM30974v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/745/GCA_000309745.1_ASM30974v1
GCA_000309765.1_ASM30976v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/765/GCA_000309765.1_ASM30976v1
GCA_000309785.1_ASM30978v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/785/GCA_000309785.1_ASM30978v1
GCA_000318035.1_ASM31803v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/318/035/GCA_000318035.1_ASM31803v1
GCA_000376145.1_ASM37614v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/376/145/GCA_000376145.1_ASM37614v1
GCA_000388095.2_LcY_assembly050913	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/388/095/GCA_000388095.2_LcY_assembly050913
GCA_000400585.1_LcA_0213	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/400/585/GCA_000400585.1_LcA_0213
GCA_000418515.1_ASM41851v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/418/515/GCA_000418515.1_ASM41851v1
GCA_000472345.1_ASM47234v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/472/345/GCA_000472345.1_ASM47234v1
GCA_000474615.1_Lcasei5b_2.0	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/474/615/GCA_000474615.1_Lcasei5b_2.0
GCA_000510825.1_ASM51082v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/510/825/GCA_000510825.1_ASM51082v1
GCA_000615205.1_ASM61520v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/615/205/GCA_000615205.1_ASM61520v1
GCA_000736295.3_L._casei_Hybrid_assembly	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/736/295/GCA_000736295.3_L._casei_Hybrid_assembly
GCA_000827145.1_ASM82714v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/827/145/GCA_000827145.1_ASM82714v1
GCA_000829055.1_ASM82905v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/829/055/GCA_000829055.1_ASM82905v1
GCA_001013375.1_ASM101337v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/013/375/GCA_001013375.1_ASM101337v1
GCA_001066565.1_ASM106656v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/066/565/GCA_001066565.1_ASM106656v1
GCA_001066695.1_ASM106669v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/066/695/GCA_001066695.1_ASM106669v1
GCA_001433735.1_ASM143373v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/433/735/GCA_001433735.1_ASM143373v1
Lactobacillus fermentum	
GCA_000010145.1_ASM1014v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/010/145/GCA_000010145.1_ASM1014v1
GCA_000159215.1_ASM15921v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/159/215/GCA_000159215.1_ASM15921v1
GCA_000162395.1_ASM16239v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/162/395/GCA_000162395.1_ASM16239v1
GCA_000210515.1_ASM21051v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/210/515/GCA_000210515.1_ASM21051v1
GCA_000397165.1_ASM39716v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/397/165/GCA_000397165.1_ASM39716v1
GCA_000417005.1_ASM41700v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/417/005/GCA_000417005.1_ASM41700v1
GCA_000466785.3_ASM46678v3	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/466/785/GCA_000466785.3_ASM46678v3
GCA_000472265.1_LF1_1.0	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/472/265/GCA_000472265.1_LF1_1.0
GCA_000477515.1_Reference_Assembly	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/477/515/GCA_000477515.1_Reference_Assembly

Table 5-7 Genomes used for multiple genome alignment

Genome Accession	NCBI FTP site
GCA_000496435.1_LfermNB22_1.0	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/496/435/GCA_000496435.1_LfermNB22_1.0
GCA_000966835.1_ASM96683v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/966/835/GCA_000966835.1_ASM96683v1
GCA_001010185.1_ASM101018v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/010/185/GCA_001010185.1_ASM101018v1
GCA_001010245.1_ASM101024v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/010/245/GCA_001010245.1_ASM101024v1
GCA_001039735.1_LFE2	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/039/735/GCA_001039735.1_LFE2
GCA_001077025.1_ASM107702v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/077/025/GCA_001077025.1_ASM107702v1
GCA_001297025.1_ASM129702v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/297/025/GCA_001297025.1_ASM129702v1
GCA_001297905.1_ASM129790v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/297/905/GCA_001297905.1_ASM129790v1
GCA_001368755.1_LF_newbler2.7	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/368/755/GCA_001368755.1_LF_newbler2.7
GCA_001436835.1_ASM143683v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/436/835/GCA_001436835.1_ASM143683v1

5.6.1.2 Strain-specific primer design and confirmation

Strain-specific primers were designed targeting to the corresponding unique sequences using PrimerQuest Tool (IDT, California) (Table 5-8 below). The specificity of the candidate primers was confirmed by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and colony PCR (Figure 5-6 below).

Table 5-8 Strains specific primer used for HRM-qPCR analysis[21]

Strains	Genome Coordinate	Primer sequence (name)	Amplicon Length (bp)	Annealing Temp. (℃)
L. casei K9-1	1720000…1730000	GTATTCGAGAACTCGGGTTGAG (LC-f1) TTACGCCTGATGACTGGATTG (LC-r1)	101	62
	1615000…1620000	CCGATGTCTTGCCGACTTTA (LC-f2) GGATCTACGGTTGCGTGAAT (LC-r2)	104	62
	1592000…1598000	GTTGGAGGATCGCGGATTAG (LC-f3) CGTCACCGGAAGTGATGTT (LC-r3)	98	62
	1541500…1542500	CCATCAAGAGGAGGCCAATAAA (LC-f4) CGCGACTATGAGCGAATGAATA (LC-r4)	109	62
L. fermentum K9-2	1180000…1184500	CTTGCTGTGATTGCCTACTTTG (LF-f1) CTTCCCTTTCTTGAGGAGTGAG (LF-r1)	91	62
	1180000...1184500	GTTTCCGCATTTCTGCTAACC (LF-f2) CCAATACCAAGCCGAACATAAC (LF-r2)	104	62
	1180000…1184500	CCCACGAGATTGCCCATATT (LF-f3) GAAGATCCATTGCCGTTTCATTAG (LF-r3)	111	62

[21] Contribution by Weilan Wang

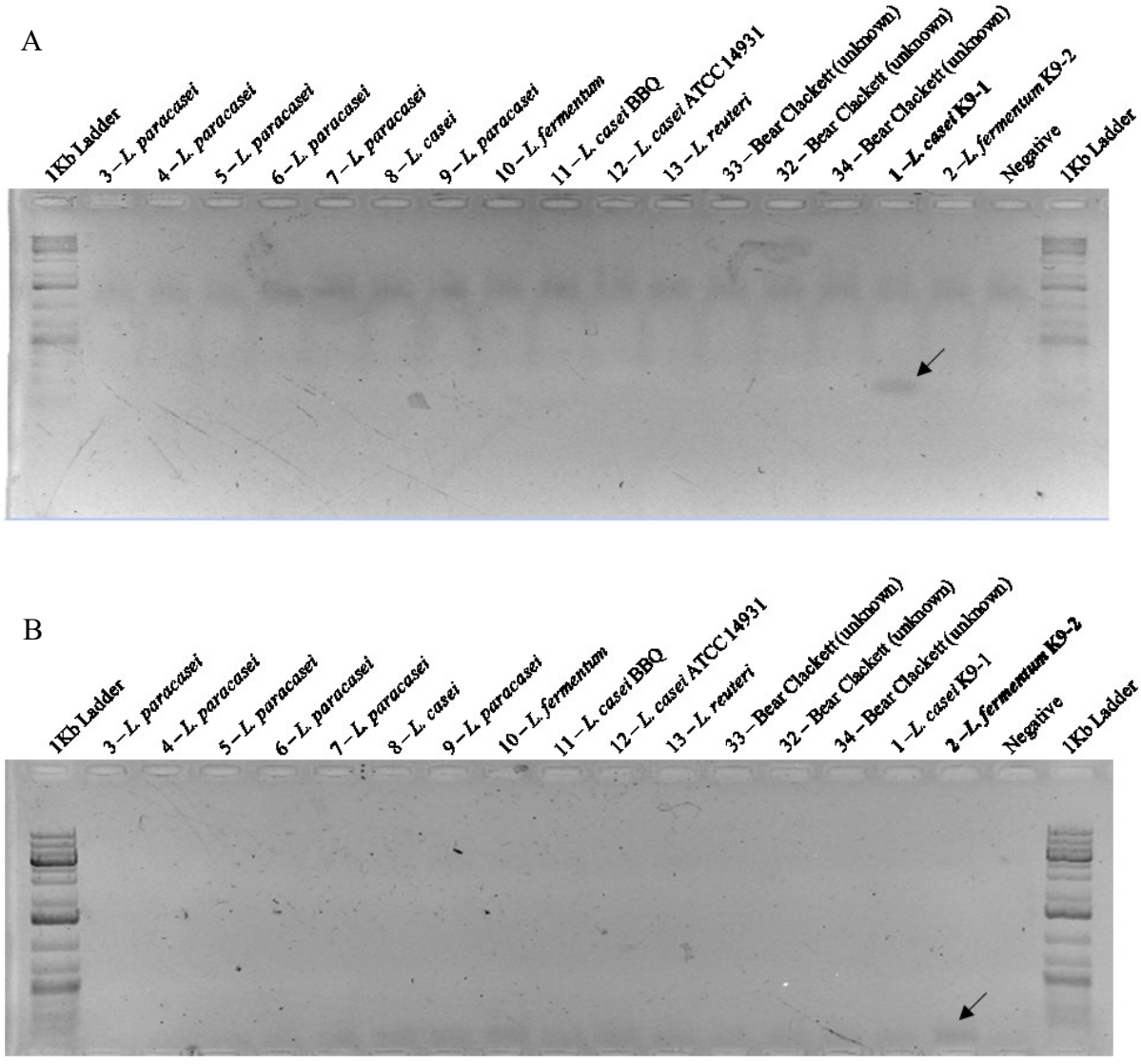

Figure 5-6 Visualization of gel electrophoresis for PCR amplicons of the strain-specific primers of *L. casei* K9-1 (A) and *L. fermentum* K9-2 (B)[22]

[22] Contribution by Alysson Blaine, and Sarah Miyata-Kane

5.6.2 Animal experimental design

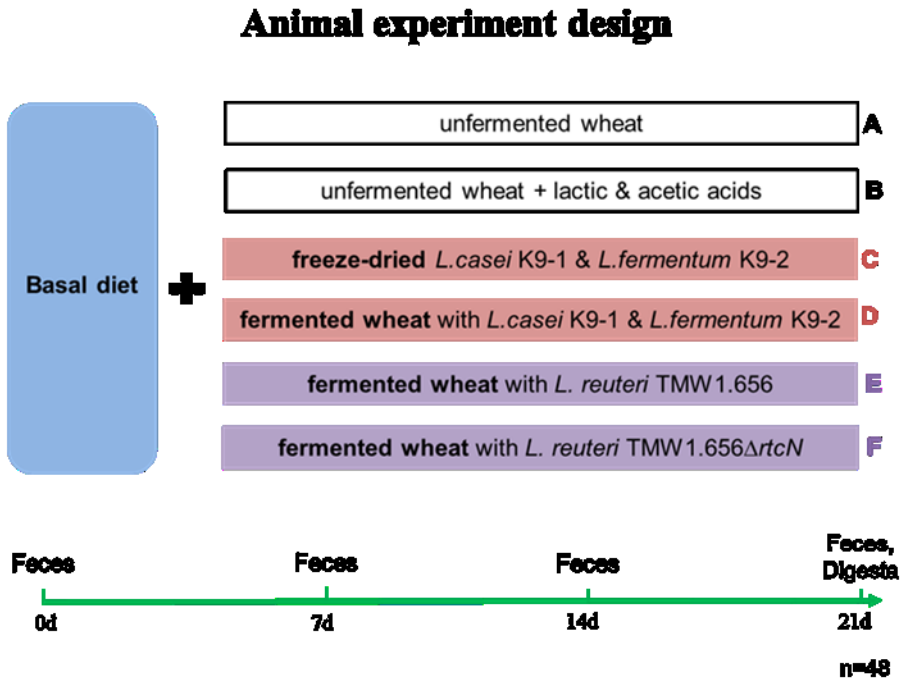

Figure 5-7 Overview on the animal experimental design

Animals were started on the experimental diets at day 1 after weaning.

5.6.3 Fecal microbiota composition[23]

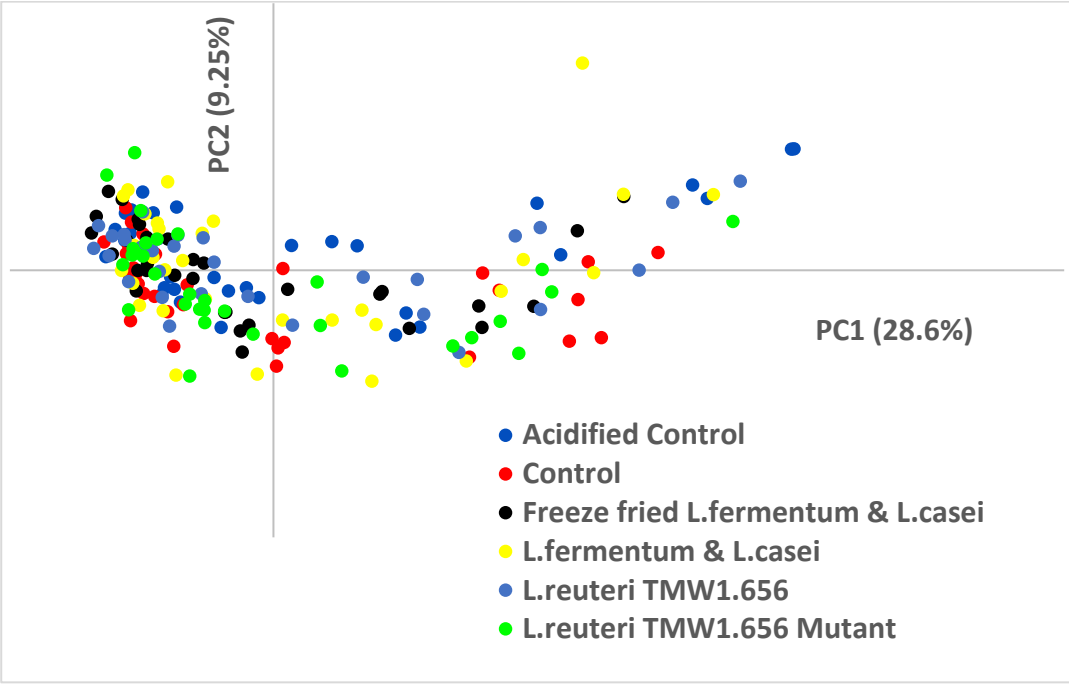

Figure 5-8 Principle Coordinate Analysis (PCoA) of fecal microbiota composition.

The analysis is based on unweighted unifrac distance of rDNA sequences (total of 6,647,893 sequences with an average length of 266 bp, corresponding to an average 34,805 sequences per sample). No significant correlation ($p = 0.122$, $R = 0.008$) between diets and fecal microbial composition was detected by Analysis of Similarities (ANOSIM).

[23] Contribution by Weilan Wang

CHAPTER 6 General discussion and future directions

The selection criteria of probiotics recommended by the working group of FAO/WHO include: i) resistance to gastric acidity, ii) antimicrobial activity against potentially pathogenic bacteria, iii) human origin (FAO/WHO, 2002). These probiotic selection criteria, however, are currently debated (Dunne et al., 2001; Hill et al., 2014; Morelli, 2000; Sanders, 2008; Vinderola et al., 2017). Morelli (2000) argued that *in vitro* acid resistance assay cannot indicate probiotic efficacy *in vivo.* Some commercial probiotic lactobacilli including *L. delbrueckii*, *L. casei*, *L. rhamnosus* and *L. plantarum* are not autochthonous to animal or human intestines, even if they were isolated from animal or human feces. Duar et al. (2017) argued that probiotic selection criteria should be re-visited to include the ecological origin of probiotic strains as an important criterion.

Lifestyles defined in lactobacilli (i.e., 'free-living', 'nomadic', and 'host-adapted') provide a framework to evaluate their autochthony to gut and suitability for the probiotic application (Duar et al., 2017). Host-adapted *Lactobacillus* species have a stable ecological association with animals and human; the nomadic *Lactobacillus* species often migrate across the animal, human, environmental and man-made habitats with a lack of niche-specific metabolic traits; the free-living *Lactobacillus* species have an ecological association with the environmental sources. Experimental evidence suggest that lifestyle of probiotics influences their their capability to permanently colonize vertebrate hosts (Duar et al., 2017a; Frese et al., 2012; Maldonado-Gómez et al., 2016; Walter et al., 2008b). Ecological lifestyles thus should be taken into consideration for the selection of probiotic lactobacilli.

Animal experiments and biochemical analysis confirmed that rodent-lineage sourdough isolates of *L. reuteri* colonize the mouse gut (Su et al., 2012; Walter et al., 2008b). In particular, lineage-

specific metabolic traits, glycerol dehydratase and glutamate-dependent acid resistance confer ecological advantages in sourdough fermentations to the *L. reuteri* strains of the respective host-specific lineages (Frese et al., 2011; Lin et al., 2014). **Chapters 3 and 4** provided a detailed genomic comparison of rodent-lineage sourdough isolates and intestinal isolates of *L. reuteri* to determine whether sourdough-specific genetic traits can be identified. Sourdough strains of *L. reuteri* and intestinal strains of *L. reuteri* do not differ with respect to their carbohydrate fermentation patterns (**Chapter 3**) but may become divergent after longer time of natural selection (**Chapter 4**). Animal experiment demonstrates that sourdough strains of *L. reuteri* survive the piglet gastrointestinal tract with significantly higher persistence in the gut relative to the free-living *L. fermentum* and nomadic *L. casei* strain (**Chapter 5**). Host-adapted lactobacilli are dominant in various fermented foods; *L. reuteri* as an example of host-adapted lactobacilli thus can guide to further explore the role of food-derived lactobacilli as an additional source of probiotics.

6.1 Sourdough isolates of *L. reuteri* are indistinguishable from rodent isolates in relation to carbohydrate utilization and genomic background

The occurrence of *L. reuteri* in the human-made habitat sourdough provides the opportunity to study the adaptation of vertebrate symbionts to an extra-intestinal habitat. This study employed comparative genomics of 16 strains of *L. reuteri* to evaluate this adaptation or selection process (**Chapter 4**). A core genome phylogenetic tree grouped *L. reuteri* into 5 clusters corresponding to the host-adapted lineages I, II, III, IV, and VI (**Chapter 4**). The topology of a gene content tree that includes accessory genes was different from the core genome phylogenetic tree, indicating gene loss or acquisition of genes by horizontal genetic transfer. Sourdough specific genes were

not identified when considering that the sampling of strains was biased for strains producing reutericyclin. A total of 124 core genes were found to under positive selection, representing about 10% of the core genome (**Chapter 4**). In the lineage III sourdough isolates, 177 genes were under positive selection, indicating that the selective pressure in sourdough differs from the selective pressure in intestinal habitats (**Chapter 4**). Positively selected proteins are significantly enriched in three COG functions, energy production, carbohydrate metabolism and defense mechanisms. Of these proteins, alcohol dehydrogenase and lactate dehydrogenase have been demonstrated experimentally to be associated with an increased growth rate of *L. reuteri* strains in sourdough fermentations. Proteins under the positive selection are not functionally different but these proteins suggest differences in selective pressure between sourdough and the rodent forestomach.

This study provided a molecular explanation for differential preference for glucose and oligosaccharide utilization in homofermentative and heterofermentative lactobacilli. This difference is regulation of carbohydrate metabolism facilitates the co-existence of these organisms in resource-restricted niches. *Lactobacillus* strains gain metabolic energy mainly from carbohydrate fermentation. Homofermentative and heterofermentative lactobacilli exhibit characteristic differences in carbohydrate transport and regulation of metabolism. However, enzymes for carbohydrate transport in heterofermentative lactobacilli are poorly characterized. This study identified carbohydrate active enzymes in the *L. reuteri* strains LTH2584, LTH5448, TMW1.656, TMW1.112, 100-23, mlc3, and lpuph by phenotypic analysis and comparative genomics (**Chapter 3**). Sourdough and intestinal isolates of *L. reuteri* displayed no difference in the number and type of carbohydrate-active enzymes encoded in the genome (**Chapter 3**). Predicted sugar transporters encoded by genomes of *L. reuteri* strains were secondary carriers mostly belonging to the major facilitator superfamily. The quantification of gene expression

during growth in sourdough and in chemically defined media corresponded to the predicted function of the transporters MalT, ScrT and LacS as carriers for maltose, sucrose, and lactose or raffinose, respectively (**Chapter 3**). The genotype for sugar utilization matched the fermentation profile of 39 sugars for *L. reuteri* strains and indicated a preference for maltose, sucrose, raffinose and isomalto-oligosaccharides, which are rich in sourdough and the upper intestine of rodents. Pentose utilization in *L. reuteri* species was strain-specific but independent of the origin or phylogenetic position of isolates. In conclusion, the study identified the lack of PTS systems, preference for secondary carriers for carbohydrate transport, and absence of carbon catabolite repression as characteristic features of the carbohydrate metabolism in the heterofermentative *L. reuteri.* Moreover, sourdough strains and rodent strains of *L. reuteri* have comparable carbohydrate resource patterns. Plant-related sugars, maltose, sucrose and raffinose are preferred both by sourdough and rodent strains of *L. reuteri*, reflecting availability of carbohydrate sources in sourdough and forestomach ecosystems.

6.2 Sourdough isolates of *L. reuteri* survive and persist in the piglet gastrointestinal tract

The stomach is the first barrier to ingested microorganisms including probiotics and enteric pathogens (Yang et al., 2013). The proton pump inhibitor treatment leads to an elevation in gastric pH, improves susceptibility of patients to enteric infections (Bavishi and DuPont, 2011; Smith, 2003), and influences gut microbiota (Imhann et al., 2015; Lo and Chan, 2013; Lombardo et al., 2010). Such a phenomenon is the direct indication that gastric acidity plays a role in eliminating ingested organisms from the host. The probiotics and pathogens possess overlapping but different acid resistance mechanisms (**Chapter 2**). Glutamate decarboxylase system and urease are

universal among enteric pathogens and probiotic bacteria. The role of particular acid resistance mechanisms in the stomach depends on species and host factors.

In vitro acid challenge conditions vary from studies to studies (**Chapter 2**). Since gastric pH of human is not constant, acid resistance phenotype assessed by traditional *in vitro* methods may not be an accurate indication of the ability of ingested bacteria to survive intestinal transit (**Chapter 2**). In addition, foodstuff will extend the empty time and temporarily increase pH in the stomach, resulting in the high survival of ingested bacteria in gastric transit. *In vitro* acid resistance cannot be extrapolated to viability in animals or human subjects (**Chapter 2**). Considering that high dose probiotics administered may compensate for the loss of viability of probiotics during gastric transit (**Chapter 2**). Therefore, we argue that *in vitro* acid resistance assessment is not necessary for the selection criteria of probiotics.

This study characterized the ecological role of reutericyclin in the back-slopped sourdough (**Chapter 4**) and the weaned piglet model (**Chapter 5**). In back-slopped sourdough, the reutericyclin positive strain of *L. reuteri* and a reutericyclin mutant has comparable competitiveness, suggesting the ecological advantage of reutericyclin is equal to the cost of reutericyclin production (**Chapter 4**). Production of antimicrobial compounds is considered to be a significant probiotic trait. The *in vivo* function, however, lacks confirmative evidence (Dobson et al., 2012; Riboulet-Bisson et al., 2012). The present study demonstrated that reutericyclin producing strain *L. reuteri* TMW1.656 persist better in piglet stomach compared to a reutericyclin deficient mutant (**Chapter 5**). Moreover, at day 14, the abundance of total lactobacilli was significantly reduced by reutericyclin producing strain. Taken together, the reutericyclin production is a significant but minor contributor to ecological fitness of *L. reuteri* in sourdough and the gastrointestinal tract.

In addition, this study suggested that the use of fermented feed may be equal or better than freeze-dried probiotic cultures with respect to intestinal persistence and performance (**Chapter 5**). The reasonable hypothesis is that feed fermentation may initiate acid tolerance of probiotic organisms before entry into the gastrointestinal tract. The effect of delivering format on the probiotic activity is species dependent.

The host-adapted *L. reuteri* persisted better in the gastrointestinal tract of weaned piglets compared to either free-living or nomadic probiotic organisms (**Chapter 5**). Despite high relatedness with other members of *L. reuteri* groups, *L. fermentum* uniquely undergoes reverse evolution from host-adapted to free-living, a common phenomenon observed in environmental symbionts (Duar et al., 2017). *L. delbrueckii*, closely clustered with other host-adapted species, also exhibits such a reverse evolution pattern resulting in restriction of *L. delbrueckii* to the dairy environment (Van de Guchte et al., 2006). *L. plantarum* and *L. casei* are both nomadic species which are generally characterized by the ability to migrate diverse ecological niches and lack of niche-specific traits. Therefore, we recommend including the lifestyle of lactobacilli into the selection criteria of probiotics.

6.3 Fermented foods provide an excellent source of probiotic lactobacilli

This thesis research infers that the ecological origin of probiotic strains is associated with transit persistence in the gastrointestinal tract of the piglet. In particular, host-adapted *L. reuteri* displays better persistence capability in pig gut compared to nomadic *L. casei* and free-living *L. fermentum* strains (**Chapter 5**). *L. casei* and *L. fermentum* strains can also be found in human and animal feces; isolation from intestinal or fecal samples, however, does not imply that isolates are

autochthonous to the human and animal gut. Therefore, consideration on ecological origin rather than source of isolation is highly recommended for probiotic screening in a future application.

Many food-fermenting lactobacilli have an origin of human and vertebrate intestine, such as *L. reuteri*, *L. panis*, *L. johnsonii*, *L. acidophilus*, *L. helveticus*, and *L. salivarius* (Tamang et al., 2016). The naturally fermented foods largely by *Lactobacillus* has a long history of safe consumption (Bernardeau et al., 2006). Fermented foods thus represent an important reservoir of probiotic lactobacilli. Food fermented with host-adapted strains and food fermented with nomadic strains both contain probiotic organisms that may differ in probiotic properties. There is limited evidence demonstrating the host adaptation at species level. However, the host-related traits of *L. reuteri* may play an important role in probiotic applications. Examples include histamine, reuterin and GABA produced by *L. reuteri* strains of respective host-adapted linages. Better understanding potential probiotic properties for human- and animal-adapted lactobacilli requires more efforts in future.

6.4 Conclusion

This thesis demonstrates that sourdough strains and rodent strains of *L. reuteri* are indistinguishable in carbohydrate utilization patterns. There is no sourdough-specific accessory genes in *L. reuteri* isolates studied in this thesis. Sourdough strains and intestinal strains including rodent strains have undergone divergent positive selection which may lead to species diversification in food fermentations that are maintained by continuous propagation over a timescale that supports speciation. The evolutionary time scale for the species diversification of *L. reuteri* was estimated to be a few millions of years (Duar et al., 2017a). As *L. reuteri* strains involved in sourdough fermentations have a vertebrate-adapted lifestyle, they survive better in the

piglet gastrointestinal tract compared to the nomadic *L. casei* strain and free-living *L. fermentum* strain, inferring that lifestyle of lactobacilli may influence their persistence in the gut ecosystem. In addition, the review of literature data indicates that *in vitro* acid resistance of probiotic strains is not firmly correlated with *in vivo* viability and probiotic performance, and thus should be excluded from the selection criteria for probiotic strains. Reutericyclin produced by *L. reuteri* strains has a non-significant impact on opportunity pathogens in weaned piglets. Antimicrobial compounds production of probiotic strains to limited degree influence autochthonous microbiota. Thus bacteriocin production is not an essential attribute for probiotic strains.

6.5 Future directions

Firstly, the animal trial suggests that the evolutionary history and ecological background may be associated with surviving and persistence of probiotic *Lactobacillus* strains in the animal and human gastrointestinal tract. In order to test this hypothesis, more strain replicates for respective lifestyles need to be applied in the animal trial as the gut ecosystem is substantially complex. Given gut microbiota is individualized, the effect to individual animal is worthwhile to be taken into consideration when assessing the interaction of probiotic strains with the host. Secondly, the current thesis focused on the influence of probiotics on the gut microbiota in the piglet gastrointestinal tract. Sourdough-derived strains of *L. reuteri* have the ability to survive and transiently persist in the piglet gut microbiota. The next question would be whether sourdough strains of *L. reuteri* confer any health benefit to the animal host. Thirdly, this present thesis demonstrates that sourdough strains of *L. reuteri* with a long association with animal and human is suitable probiotics. Provided that many types of fermented foods contain *Lactobacillus* species autochthonous to the animal or human gastrointestinal tract, we will attempt to screen for lactobacilli with a host-adapted lifestyle present in the fermented foods other than sourdoughs.

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