Host-adapted lactobacilli: evolution, molecular mechanisms and functional applications

by

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ABSTRACT

Bacteria of the genus *Lactobacillus* can be found associated with plants, insects and vertebrate hosts, and their lifestyle can range from free-living to strictly host specific. Of the lactobacilli associated with vertebrates, the lifestyle of *L. reuteri* is particularly well understood. The species has been studied by population genetics, comparative genomic and functional analyses in animal models. The phylogenetic structure of *L. reuteri* suggests that lineages evolved alongside with rodents, poultry, swine and humans. For rodent strains, co-evolution resulted in host-adaptation. The first goal of this dissertation was to determine whether host-adaptation extended to non-rodent lineages and also to resolve open questions regarding the evolutionary relationships within lineage VI, which is shared by human and poultry isolates. An experimental approach was devised to determine the ability of strains to propagate under the ecological conditions of the gastrointestinal tract (GIT) of different hosts. Rodent isolates became enriched in the GIT of mice and poultry isolates in chickens. Moreover, human isolates of the lineage VI were found to be competitive in the GIT of chickens but not in humans. These findings revealed that *L. reuteri* evolved host-specialization in rodents and chicken, while open questions remain about the exact evolutionary consequences in humans and pigs.

Biofilm formation is a common strategy by which lactobacilli maintain stable associations with their hosts. Only rodent isolates of *L. reuteri* can produce biofilms in the forestomach of mice. The second goal of this dissertation was to determine the role of a rodent-specific two component system (TCS70529-30) in biofilm formation of the rat isolate *L. reuteri* 100-23. Experiments in monoassociated mice revealed that mutation of the response regulator, but not the histidine kinase impaired biofilm formation. *In vitro* experiments confirmed *in vivo* and findings and further revealed significant alterations in the architecture of the mutant biofilms. Compared to the wildtype, histidine kinase mutants produced thick and robust biofilms, while the response regulator mutants formed thinner and less adherent biofilms. These findings provide empirical

evidence of rodent specific signal transduction system playing a role in biofilm formation of *L reuteri*, likely by regulating genes responsible for development of the biofilm matrix.

Contrary to rodent strains, human isolates of *L. reuteri* lack the genetic machinery to form biofilms, but conserve a 58-gene *pdu-cbi-cob-hem* cluster (*pdu*-cluster). Encoded in the *pdu*-cluster is the PduCDE diol dehydratase involved in utilization of 1,2 propanediol (1,2 PD). In the human gut, 1,2 PD is readily available as a result of fermentation of rhamnose and fucose found in dietary and host-derived glycans, respectively. The third goal of this dissertation was to determine the role of the *pdu*-cluster in utilization of 1,2 PD by human isolates of *L. reuteri.* The ability of the human isolate *L. reuteri* ATCC 6475 to cross-feed from 1,2 PD produced by *Escherichia coli* MG1655 and *Bifidobacterium breve* UCC2003 was determined *in vitro* and compared to a *pduCDE* mutant. We found that during fermentation of hexoses, 1,2 PD serves as an electron acceptor increasing the metabolic efficiency of *L. reuteri*, a factor that could be pivotal to the competitiveness of human isolates of the human GIT.

The fourth goal of this dissertation was to identify and characterize bacterial isolates from the proximal GI tract of pigs capable of degrading peptides involved in the etiology of celiac disease. Strains were selected from the GIT tract of pigs fed a 20% gluten diet and after an *in vitro* process aimed to enrich for gluten degrading bacteria. Pigs were selected as these animals harbor large amounts of lactobacilli. Strains of the species *L. amylovorus, L. johnsonii, L. ruminis, and L. salivarius* were identified as having the highest proteolytic activity against several well characterized gluten immunotoxic peptides. Since these strains are adapted to the conditions in the proximal GI tract, they are likely to be good candidates for probiotics aimed at removing gluten epitopes before they reach the epithelium of the small intestine in celiac patients.

Together findings in this dissertation contribute to our understanding of the evolution of *L. reuteri* with different vertebrate hosts, reveal insights into lineage-specific functions underlying adaptation to the vertebrate GIT, and provide a basis for the selection of lactobacilli adapted to GIT for functional applications.

PREFACE

This thesis is an original work by Rebbeca M. Duar

A version of **Chapters and 6** – are part of invited review article currenty in revision (April, 2017) to be published in the journal *FEMS Microbiology Reviews* in a dedicated edition for the LAB12 conference as: R.M Duar, X B. Lin, J. Zheng, M.E Martino, T Grenier, ME Pérez-Muñoz, F Leulier, MG Gänzle, J Walter. Lifestyles in transition: Evolution and natural history of the genus *Lactobacillu*s

R.M.D and X.B.L, M.G.G and J.W contributed equally to this work by conceptualizing the idea, analyzing the data, writing and editing the manuscript. M.G.G and J.Z performed the phylogenomic analyses. M.G.G provided the type strains' metadata and conducted metagenomics analysis. M.E designed the illustrations M.E.M, T.G and F.L wrote the section on the nomadic lactobacilli and edited the manuscript.

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R.M.D designed the experiments, collected and analyzed the data, and wrote the manuscript. J.W designed the experiments, supervised data analyses, wrote and edited the manuscript. S.A.F designed the experiments, conducted the mouse experiments and edited the manuscript. S.E.F and T.E.B provided the gnotobiotic pig mode and gave technical advice. CQW and CMS provided the chicken model and gave conceptual and technical advice. D.A.P provided mice and gave technical advice. G.T helped preparing materials and collected data. J.B gave technical support and conceptual advice in for the comparative genomic analyses.

Chapter 3- RM. Duar, XB. Lin, T Grenier, M Bording-Jorgensen, LA. Cody, E Wine, AE. Ramer-Tait, MG. Gänzle, J Walter. A rodent-strain specific two-component system regulates biofilm formation of *Lactobacillus reuteri* 100-23. Manuscript in preparation.

R.M.D generated the mutants, designed the experiments, collected and analyzed the data, and wrote the paper. X.B.L provided technical advice during the generation of the mutants and conduced the *in vivo* experiments. T.G designed and conducted in *vitro* experiments and performed the SEM sample preparation and image collection.. E.W provided materials for CLSM. M.B.J provided technical advice for confocal microscopy, collected the images and conduced part of the CLSM image collection. L.A.C conduced the confocal imaging analysis of the *in vivo* samples. A.E.R provided mice and supervised mouse experiments at the University of Nebraska. M.G.G provided conceptual and technical advice. J.W conceived the study, conceptualized the experiments and supervised data analysis.

Chapter 4 -RM. Duar, C. Cheng, XB. Lin, S Mohamed, JP van Pijkeren, JH Oh, D Van Sinderen, MG. Gänzle, J Walter. *In vitro* cross-feeding of 1,2 propanediol in human isolates of *Lactobacillus reuteri*. Manuscript in preparation.

R.M.D conceived the study, designed the experiments, collected and analyzed the data, and wrote the manuscript. C.C and S.M conducted the experiments and collected data. X.B.L provided conceptual advice and performed HPLC analysis. J.P and J.H.O generated the mutant. D.V.S provided the strain *Bifidobacterium breve* UCC2003 and gave technical and conceptual advice. MGG and J.W conceptualized the experiments and supervised data analysis.

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R.M.D conducted experiments, collected and analyzed data, and wrote the manuscript. K.J.C, P.B.P, C.H and S.B conducted experiments, collected and analyzed the data. S.L.T provided materials, conceptual and technical advice during the ELISA analyses. T.E.B provided the pigs and gave technical advice. N.M conduced the mass spectroscopy analysis. J.W conceived the project, conceptualized the experiments, supervised data analysis, wrote and edited the manuscript.

Chapter 6- Conclusions, implications and future directions

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During my time in grad school I've developed friendships with some truly wonderful individuals, María Isabel, Andrés, Sarah (s), Rae and Mike, thanks for letting me vent, for giving me advise and for making me laugh.

I would like to dedicate my work to my whole family for understanding my dream to study abroad and for their permanent support in my life, especially to my mom and aunt, two strong and independent women I was very fortunate to have as role models. I would like to give a very special thanks to my husband Dallas, for his loving support and for putting up with me during stressful times.

"*And, when you want something, all the universe conspires in helping you to achieve it*."

-Paulo Coelho, The Alchemist

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GLOSSARY OF TERMS

Adaptation: Process by which an organism becomes more fitted to an environment as the result of natural selection.

Allochthonous: Originates from a place other than that in which it is found.

Autochthonous: A true resident, found where formed.

Dispersal: Movements of individuals from a source location to another location where establishment and reproduction may occur.

Free-living: Associated with plant material and/or environment without relying on an eukaryotic host.

Habitat: The natural environment in which an organism lives.

Host-adapted: Specialized towards living in association with eukaryotic hosts, with adaptive traits that facilitates persistence

Lactobacillus sensu lato: **(**From Latin: “in the broad sense”). Includes the lactobacilli and related pediococci.

Lifestyle: The way of life of a species which allows its population to persist in nature.

Natural history: *A*n organism's ecological interactions in its natural habitat and how they evolved.

Niche (Hutchinsonian niche)**:** Environmental conditions and resources within which a species can maintain a viable population

Nomadic: Dynamic lifestyle that involves both environmental and host niches, with no signs of specialization.

Specialized: Restricted in the breadth of its ecological niches as a result of trade-offs during adaptation**.**

Symbiosis (From Greek: *sym* “with” and *biosis* “living”) Long-term associations between genetically distinct organisms

1. Chapter One: Evolution and lifestyles of species of the genus *Lactobacillus*

1.1 Introduction

Lactobacilli are fastidious gram-positive bacteria that populate nutrient-rich habitats associated with food, feed, plants, vertebrate and invertebrate animals, and humans. Owing to their use in food, in biotechnology and in therapeutic applications, lactobacilli have substantial economic importance. Consequently, research focused on their role in food fermentations and spoilage (Chaillou *et al.* 2005; Gänzle and Ripari 2016; Stefanovic, Fitzgerald and McAuliffe 2017) biotechnological applications (Sun *et al.* 2015) and their functionality as 'probiotics', which are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Marco, Pavan and Kleerebezem 2006; Lebeer, Vanderleyden and De Keersmaecker 2008; Bron, van Baarlen and Kleerebezem 2011; Hill *et al.* 2014). These studies have provided important information regarding the metabolism and functionality of a wide array of *Lactobacillus* species in the food environments and gastrointestinal tract, and their role in human and animal health. From an ecological and evolutionary perspective, however, these studies provide little insight as they are conducted in experimental settings that are abstracted from any natural history. Food habitats are man-made and date back less than 14,000 years (Steinkraus 2002; Hayden, Canuel and Shanse 2013) which is short when considering that the natural history of lactobacilli with plants and animals dates back more than a billion years (Tailliez 2001; Battistuzzi *et al.* 2004). Furthermore, most probiotic research has been conducted with *Lactobacillus* strains 'allochthonous' to the respective hosts in which they were studied (Walter 2008). We therefore lack information regarding the evolution of lifestyles in lactobacilli as it occurred in their true ecosystems in nature.

The genus *Lactobacillus* comprises more than 200 species that are characterized by a phylogenetic and metabolic diversity that exceeds that of a typical bacterial family (Sun *et al.* 2015). Recent phylogenetic analyses based on robust core genome phylogeny have revealed that lactobacilli can be subdivided into at least 24 phylogenetic groups (Zheng *et al.* 2015a); species of the genus *Pediococcus* form an integral part of the genus *Lactobacillus.* Accordingly, lactobacilli have been referred to as the *Lactobacillus sensu lato* including pediococci, or the *Lactobacillus* Genus Complex to additionally include the related genera *Weissella, Leuconostoc, Oenococcus* and *Fructobacillus* (Sun *et al.* 2015; Zheng *et al.* 2015a). The availability of genome sequences of lactobacilli has created a robust framework for large scale phylogenomic and comparative genomic analyses that can elucidate their evolution (Sun *et al.* 2015; Zheng *et al.* 2015a). In addition, population genomic and genetic analyses have allowed a detailed reconstruction of the evolutionary patterns in specific *Lactobacillus* species (Oh *et al.* 2010; Frese *et al.* 2011; McFrederick *et al.* 2013; Martino *et al.* 2016). If informed by an understanding of the metabolic traits of *Lactobacillus* groups and lineages, these analyses provide an opportunity to explore the ecological and evolutionary contexts in which these bacteria exist in nature and how their lifestyles have evolved.

This review compiles the available genomic and metabolic metadata for the genus *Lactobacillus* to infer its evolution and natural history. Specifically a phylogenomic approach is applied to infer the natural habitat and then related to metabolic, functional and fine-scale phylogenetic analyses of model species. Lastly, remaining questions and how research in this dissertation aimed to address these questions is discussed.

1.2 Habitats of lactobacilli

Restricted by fastidious growth requirements, lactobacilli occupy nutrient-rich habitats which can be categorized into fermented or spoiled foods and animal feed, the environment including plants surface, soil, and the body of invertebrate and vertebrate animals (Fig. 1.1).

1.2.1.1 Food and feed

Lactobacilli dominate the microbiota of the vast majority of fermented foods and also occur as food spoilage organisms (Hammes and Hertel 2006; Gänzle 2015). Fermentation of silage, vegetables and many cereals relies on the microbiota of the raw materials as source of inoculum. Other fermentations, including most dairy fermentations, sourdough, and fermented meats are controlled by back-slopping or "house microbiota" that are associated with the production environment (Scheirlinck *et al.* 2009; Su *et al.* 2012; Chaillou *et al.* 2013; Ripari, Gänzle and Berardi 2016). Organisms in these fermentations are exposed to continuous propagation over decades or even centuries, essentially becoming domesticated to the fermentation environments (van de Guchte *et al.* 2006; Vogel *et al.* 2011; Ding *et al.* 2014). Adaptation to conditions in food fermentations was suggested for *L. delbrueckei* ssp. *bulgaricus*, which shows rapid and ongoing reduction of the genome size (van de Guchte *et al.* 2006). However, genomic analysis of intestinal and sourdough isolates of *L. reuteri* indicated differential selective pressure in the two environments but not phylogenetic differentiation (Zheng *et al.* 2015b). The majority of the type strains of the *Lactobacillus* species have bee isolated from food (Fig1.1 a); however, food fermentations are unlikely to represent the primary habitat for *Lactobacillus* spp. (Fig. 1.1 b).

1.2.1.2 Environmental sites and plants

Lactobacilli occur frequently in sewage as a result of fecal contamination and occasionally in soils as part of the rhizosphere of plants or as a result of wash-off from the phyllosphere (Kvasnikov, Kovalenko and Nesterenko 1983; Hammes and Hertel 2006). Despite the occasional reports of lactobacilli being isolated from wheat, beet and strawberries (Jacobs, Bugbee and Gabrielson 1985; de Melo Pereira *et al.* 2012; Minervini *et al.* 2015), lactobacilli are a rare and minor component of the plant endophytes (Hallmann *et al.* 1997). Lactobacilli are detected in small numbers on plant surfaces, where traces of sugars can support their growth (Mercier and Lindow 2000). Their numbers only increase upon damage of plant tissue when simple and

complex carbohydrates become available substrates (Müller and Lier 1994). The ecological role of plant-associated lactobacilli in nature is poorly understood, but because their occurrence is only sporadic, they are not considered plant symbionts but rather epiphytic (Stirling and Whittenbury 1963; Mundt and Hammer 1968; Fenton 1987).

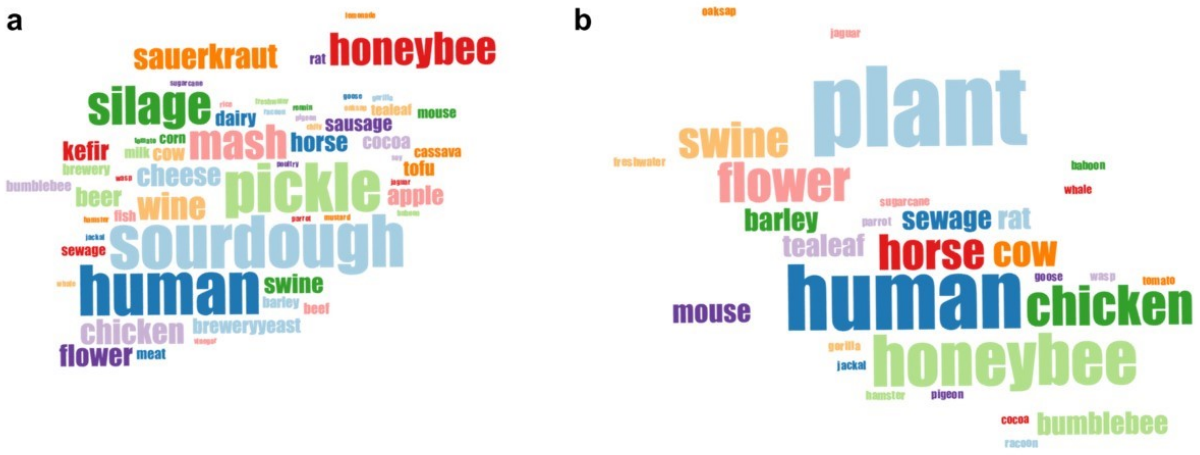


Figure 1.1- Word cloud representing the origin of lactobacilli.

The words describe the source of isolation of the type strains of lactobacilli; the square root of the font size of the words correlates to its frequency. (a) The isolation source of the 204 type strains of lactobacilli as described by Pot et al., (2014) or the newly described species. The description was simplified as follows: All strains of human or animal origin are designated as human or animal, irrespective of the site of isolation. The origin of all isolates from cereal mashes used for production of alcoholic beverages are designated as “mash”. The origin of all isolates from flowers, vegetable, sourdough, and silage fermentations were designated as “flower”, “pickle”, “sourdough” and “silage”, respectively, irrespective of the plant species. The origin of all strains isolated from kimchi, sauerkraut, and fermented cabbage was designated as “sauerkraut”. The origin of isolates from various stages of beer, wine, and apple cider fermentation was designated as “beer”, “wine”, and “apple”, respectively. The words “poultry” and “beef” represent meat; the words “chicken” and “cow” represent animals. (b) The origin of the same 203 type strains with a further simplification of the description of the origin as follows: the words representing spontaneous plant fermentations (pickle, sauerkraut and silage” was replaced by “plant”. The origin of all other food-associated organisms was omitted. The word cloud was generated with the online tool available at https://wordsift.org/.

1.2.1.3 Vertebrate and invertebrate hosts

Lactobacilli are reliably isolated from a variety of insects including flies and bees, and from vertebrates, particularly birds, rodents, humans and farm animals. The host range is likely larger as scientific investigations have been largely restricted to domesticated animals and humans (Endo, Futagawa-Endo and Dicks 2010; McFrederick *et al.* 2013; Martino *et al.* 2016). Food storage organs such as the forestomach and crop appear to be the preferred habitat of lactobacilli in animal hosts. These organs are found in both insects (flies, bees, bumblebees) and vertebrate animals (poultry, rodents). In humans, lactobacilli are found in the oral cavity, gastrointestinal tract, with highest proportions in the small intestine and the vagina (Walter 2008).

1.3 What are the lifestyles of lactobacilli in nature?

Although we have a comprehensive knowledge of the origin of *Lactobacillus* strains, the precise ecological niches and lifestyles of these bacteria are difficult to unravel. To date, most functional research concerns the metabolic and, more recently, genetic adaptations to conditions that prevail in food and feed fermentations (Fig. 1.1a). However, although food fermentations provide opportunities for clonal expansion of specific species or phylogenetic groups (Cai *et al.* 2007; Chaillou *et al.* 2013; Zheng *et al.* 2015b), the adaptation of lactobacilli to these men-made habitats is coincidental and recent, and diversification, if it occurs, remains below the species level (Cai *et al.* 2007; Chaillou *et al.* 2013; Zheng *et al.* 2015b). From an evolutionary perspective, food, feed, and biotechnological fermentations cannot be considered as habitats that supported speciation and cannot be considered for the elucidation of *Lactobacillus* lifestyles (Fig. 1.1b). Although some species have been traced to animals, environment, and raw materials (Scheirlinck *et al.* 2009; Su *et al.* 2012; Chaillou *et al.* 2013; Ripari, Gänzle and Berardi 2016), the real ecological niches and natural history of most *Lactobacillus* species present in food and feed remains unknown.

Predictions about the exact natural history of lactobacilli are difficult even for organisms that are reliably found in habitats that support speciation. Lactobacilli can be 'allochthonous', meaning, they originate from a different place, and have, in contrast to 'autochthonous' species, neither an ecological nor evolutionary relationship with the habitat in which they are found. This concept is especially relevant for the gastrointestinal tract of humans where lactobacilli originate from fermented food (Tannock 2004; Walter 2008), but also relates to other habitats including wastewater, plants, flowers, and nectar, where lactobacilli may be present as fecal contaminants from vertebrates or insects. Autochthonous organisms establish long-term and stable populations of typical sizes and exert specific ecological functions in the habitat (Tannock 2004). However, even if such conditions are met, conclusions regarding the natural history of a species have to be drawn with caution. Allochthonous species establish stable populations when being introduced regularly into a habitat, and they may exert ecological functions even if such habitats are irrelevant to their evolution, as is the case of fermented foods. In addition, habitats or hosts that only allow sporadic and transient colonization may still play an important role in the overall lifestyle of a species, for example by providing vectors for dispersal or a temporal refuge (Vellend 2010). It conceivable that species possess a dynamic lifestyle comprised by more than one stable niche in which a classic autochthony could evolve.

Given these complexities, a combination of complementary approaches is required to reliably elucidate the natural history of lactobacilli. In the following sections the lifestyles of *Lactobacillus* species are deduced by synthesizing phylogenomic data with information on the metabolism of the bacteria, and inform these inferences with findings from more focused population genetics and functional studies. Specifically, (*i*) lifestyles are assigned based on a phylogenetic context, considering factors such as occurrence and frequency of detection/isolation as well as the strains' metabolic characteristics and their ability to withstand environmental stressors present in given habitats; (*ii*) the evolutionary transitions between lifestyles are investigated by using a phylogenetic approach that is conceptually similar to that described by Sachs and co-workers

(Sachs, Skophammer and Regus 2011); (*iii*) patterns of genome evolution described to be associated with the evolution of symbiotic lifestyles are analyzed (Lo, Huang and Kuo 2016); (*iv*) this overview is then complemented with findings from fine-scale population genetic and functional studies on representative species that can serve as paradigms for the specific lifestyles represented within the lactobacilli.

1.3.1 Evolutionary insight through phylogenomics

The diversification of anaerobic clostridia and aerobic or facultative anaerobic bacilli and lactic acid bacteria roughly matches the “great oxidation event” that occurred ~2.5 billion years ago (Battistuzzi *et al.* 2004). *Lactobacillales* then diverged from staphylococci and bacilli approximately 1.8 billion years ago (Battistuzzi *et al.* 2004), substantially predating the emergence of land plants (~500 million years ago), insects (~400 million years ago), mammals (~200 million years ago) and birds (~80 million years ago) (Shetty, Griffin and Graves 1999; Hedges *et al.* 2004; Luo 2007; Clarke, Warnock and Donoghue 2011; Pires and Dolan 2012; Misof *et al.* 2014). However, diversification within the genus *Lactobacillus sensu lato* likely intensified with the emergence and later diversification of the eukaryotic species with which lactobacilli became associated (Tailliez 2001).

To gain insight into lifestyle evolution of lactobacilli, we updated the core phylogenomic tree of *Lactobacillus sensu lato* (Zheng et al. 2015a) by adding species for which genome sequences became recently available (Fig. 1.2 and Table 1.S1). Based on isolation source, frequency of isolation, metabolic capabilities, growth temperature, and the ability to withstand environmental stressors present in given habitats, we assign species into three main lifestyle categories: free-living (encompassing environmental and plant isolates), host-adapted (associated with invertebrate or vertebrate hosts), or as ‘nomadic’ using the concepts proposed by Martino and co-workers (Martino et al. 2016). Remarkably, lifestyle assignments show a high correlation with phylogenetic grouping (Fig. 1.2). This association strongly suggests that monophyletic clades

within the lactobacilli are the results of adaptive evolution in different habitats, which resulted in the emergence of distinct lifestyles, with a high degree of phylogenetic niche conservation. Specifically, the *L. perolens, L. sakei, L. vaccinostercus, L. collinoides, L. brevis,* and *L. buchneri* groups are almost completely composed of species that are rarely found in animals, and are therefore likely free-living. The species in the *L. reuteri* group are consistently associated with vertebrate hosts (human oral and vaginal cavity, intestinal tract, primates, other mammals, birds), while the *L. salivarius* group contains a monophyletic cluster associated with vertebrate hosts (humans, rodents, birds, horses, cattle, swine, primates, whales) (Table S1) and a second cluster comprising mainly free-living species. The large and diverse *L. delbrueckii* group comprises clusters of species adapted to insects and to vertebrates including mammals such as pigs and hamsters and different species of birds). Species in the *L. plantarum* group and a cluster with the *L. casei* group are nomadic, being reliably found in a wide variety of niches.

The conservation in the niche assignments of the deep-branching monophyletic lineages within the lactobacilli suggests that lifestyles evolved for long periods of evolutionary time and were stably maintained. These clear associations further pinpoint how lactobacilli evolved specific lifestyles. Lifestyle transitions occurred in 6 separate events (See Fig. 1.2 and legend for details). The host adapted *L. delbrueckii, L. salivarius,* and *L. reuteri* groups likely evolved from free-living ancestors to become associated with vertebrates (events 1-3), while the *L. fructivorans*, *L. kunkeei* and *L. mellifer* groups evolved to become associated with insects (events 4 and 5). In the *L. delbrueckii* group, a cluster of species related to *L. apis* appeared to have switched hosts and evolved from vertebrate-adapted to bee-adapted (event 6). In addition, *L. fermentum* is the only species in the *L. reuteri* group which is rarely found in intestinal ecosystems but frequently isolated from plants and spontaneously fermented cereals (Mundt and Hammer 1968; Hammes and Hertel 2006; Gänzle and Ripari 2016). *L. fermentum* could be an example a species undergoing reversion of the lifestyle from host-adapted to free-living, a process that has been documented

for environmental species that cluster within phylogenetic clades dominated by symbionts (Sachs, Skophammer and Regus 2011).

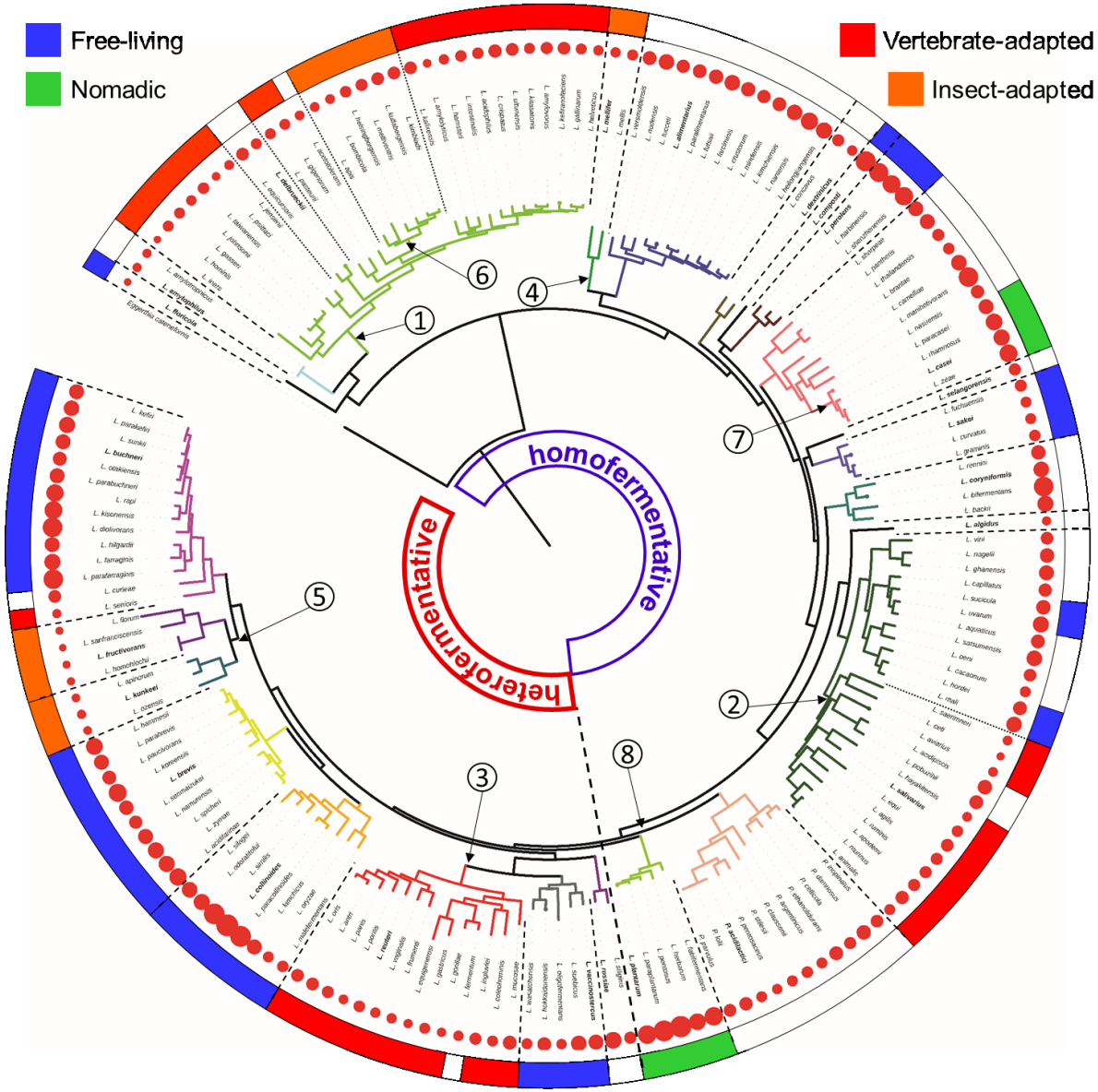
1.3.2 Patterns of genome evolution reflect an evolutionary transition to a symbiotic lifestyle

The genomes of lactobacilli range in size from 1.27 (*L. iners*) to 4.91 Mb (*L. parakefiri*) and the number of genes between species varies considerably (Sun *et al.* 2015, Table S1). Lactobacilli underwent a process of genome reduction over the course of their evolution, losing on average approximately 3000 genes from the common ancestor and 1,300–1,800 genes in individual groups or species (Makarova *et al.* 2006; Sun *et al.* 2015; Zheng *et al.* 2015a). Gene decay in lactobacilli has led to substantial loss of functions in carbohydrate metabolism, amino acid and cofactor biosynthesis, leading to the fastidious nutritional requirements of the species (Makarova *et al.* 2006). This process is especially pronounced in lactobacilli associated with animals (Sun *et al.* 2015) and been attributed to nutrient-rich environments within host habitats (Makarova *et al.* 2006). However, genome reduction is an evolutionary process that is universally observed in symbionts and directly associated with the degree of host specialization (Lo, Huang and Kuo 2016). The stable environment provided by the host renders functions that were essential in the free-living ancestor redundant, which leads to an accumulation of loss-of-function mutations and pseudogenes followed by removal of these genetic regions, e.g. through mobile genetic elements (Lo, Huang and Kuo 2016). Genome reduction is strongly correlated with host adaptation in *Lactobacillus* species, genome size is significantly lower in host-adapted but not nomadic strains (Fig. 1.3 a and b). Interestingly, genomes of host-adapted lactobacilli also show a reduction in GC content; this reduction of GC content is not observed in nomadic lactobacilli (Fig. 1.3 a and d). This constitutes another well documented pattern observed in the genome evolution and is caused by strong mutational bias toward AT and non-adaptive loss of DNA repair genes of host-

adapted symbionts (Lo, Huang and Kuo 2016). Taken together, host-association in lactobacilli correlates with genomic events that are characteristic of the evolution of a symbiotic lifestyle.

1.3.3 Metabolic capabilities reflect lifestyle adaptations

Species within the *Lactobacillus sensu lato* show a substantial degree of variation in their metabolism (Gänzle 2015). The two phylogenetic clades of lactobacilli representing homofermentative and heterofermentative organisms, however, do not reflect association to specific habitats; both homo- and heterofermentative species associate with vertebrate animals, insects, or environmental habitats (Fig. 1.2). Remarkably, many habitats harbour both homofermentative and heterofermentative lactobacilli. Examples not only include intestinal habitats including the gut microbiota of fruit flies (*L. plantarum* and *L. fructivorans* groups), bees (*L. mellifer* or *L. delbrueckii* and *L. kunkeii* groups, Anderson *et al.* 2013; Filannino *et al.* 2016) and the intestinal microbiota of vertebrate animals (*L. delbrueckii* and *L. reuteri* groups, Walter 2008) but also fermentation or spoilage microbiota in many foods including cereal fermentations, vegetable fermentations, and meat (Gänzle 2015; Hammes and Hertel 2006). Emerging evidence indicates that homo- and heterofermentative lifestyles are complementary rather than competitive (Gänzle, Vermeulen and Vogel 2007; Tannock *et al.* 2012; Andreevskaya *et al.* 2016; Andreevskaya 2017). Other differences in carbohydrate utilization patterns and growth temperature, however, provide helpful insights into niche adaptations. Free-living species are capable of growing at lower temperatures, while host-adapted species grow optimally at temperatures close to the body temperature of their corresponding hosts (Fig. 1.3e). The enzymatic repertoire of the species is also indicative of the substrates available in their natural habitats. Together, this information is essential to elucidate the exact lifestyle of the species and the characteristics of the niches to which the strains have adapted to.



The tree by was constructed according to Zheng et al. (2015) with the inclusion of 18 additional species for which genome sequence data became available since 2015. Eggerthia catenaformis was used as an outlier for the phylogenetic analysis. The inner segments delineate homofermentative and heterofermentative species, respectively. Members of the 24 phylogenetic groups are indicated by the same color for branches and the type strain of each group is printed in bold. Clusters in the *L. delbrueckii* and *L. salivarius* groups that differ in their ecology are separated by dashed lines. The solid circles in red represent genome sizes of these type strains; the area of the circle correlates with the genome size. Color coding of the outer ring indicates the lifestyle, if sufficient information is available. The habitat was assigned based on phylogenetic and ecological studies as well as literature related to the source of isolation; the assignment was additionally guided by database searches on the Integrated Microbial NGS Platform https://www.imngs.org (Lagkouvardos et al. 2016). Numbers indicate evolutionary transitions of lifestyle assuming an ancestral free-living state. Ancestral state reconstructions were executed in the Mesquite software package Version 3.2, http://mesquiteproject.org (Maddison and Maddisson 2017).

Figure 1. 2 Core genome phylogenetic tree of *Lactobacillus sensu lato* (*Lactobacillus* spp. and *Pediocccus* spp.

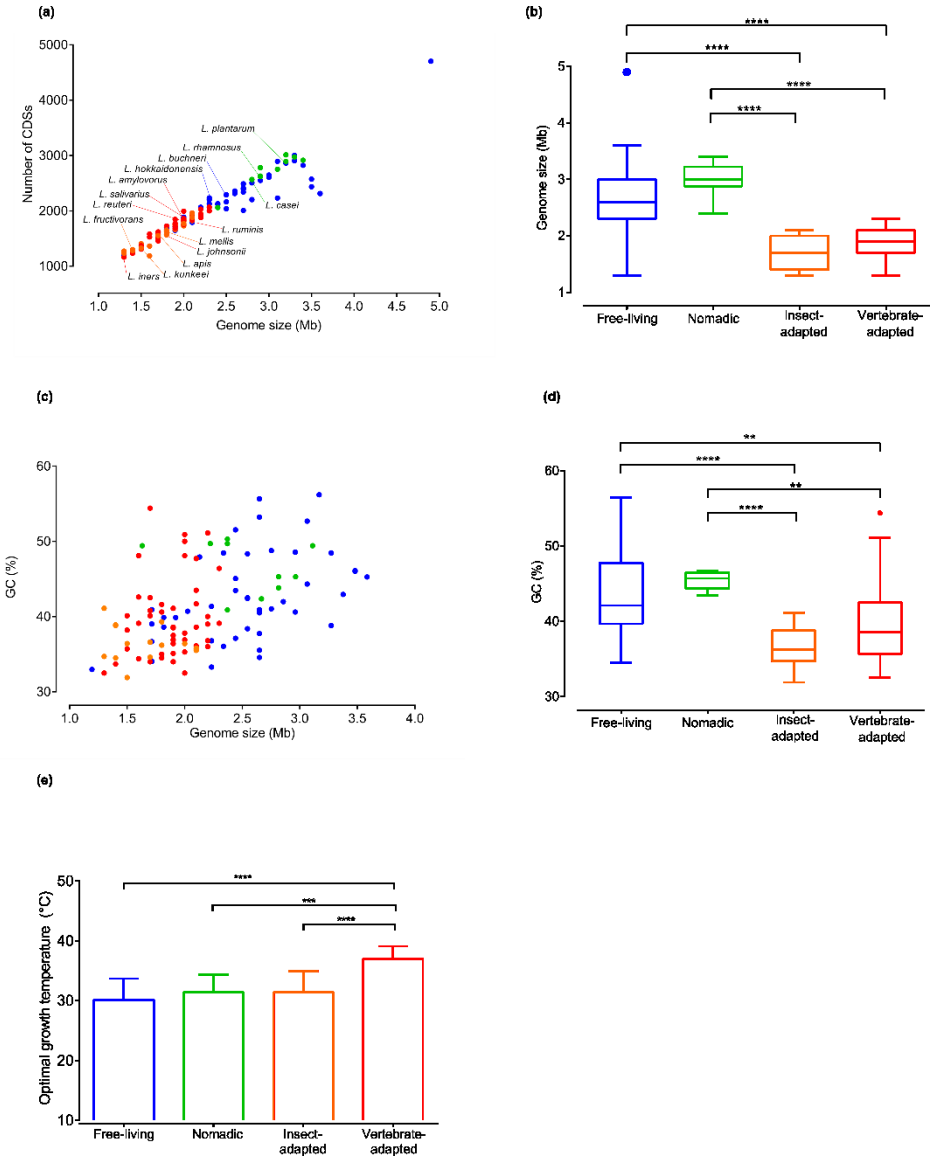


Figure 1.3 Genomic and metabolic characteristics of lactobacilli reflect different lifestyles

(a) Association between genome size and the number of coding sequences (CDSs). Pearson r = 0.95, p<0.0001. (b) Comparison of genome size (Mb) by lifestyle. (c) Association between GC content (%) and the number of CDSs. Pearson r = 0.58, p<0.0001. (d) Comparison of GC content (%) by lifestyle (E) Comparison of optimal growth temperature (mean ± SD) by lifestyle. Information was obtain from the genomes of type strains (Table S1). Representative species in panels A and C are color coded by lifestyle using the colors from Fig. 1.2. Box plots in panels B and D represent the median and the lower and upper quartiles. Whiskers extend to the last data point still within 1.5 inter-quartile range of the quartile. Kruskal–Wallis with a Dunn's post hoc test was used to compare data between groups. Statistical significant groups are indicated (***, p<0.001; ****, p≤ 0.0001). Statistical analyses were performed in GraphPad Prism version 6.07 (GraphPad Software, La Joya, CA, USA).

1.4 Paradigms of *Lactobacillus* lifestyles

1.4.1 Free-living lifestyle

Species that are found in plant and environmental sources are scattered around the phylogenetic tree (Fig.1.2), which suggests an environmental ancestral condition for the most recent common ancestor of the genus. Free-living lactobacilli are clustered in the *L. buchneri*, and *L. collinoides* groups, and all the species in the *L. brevis, L. composti and L. perolens* groups (Fig. 1.2).

Although it is difficult to determine if a lifestyle is strictly free-living, this lifestyle is strongly suggested by several characteristics of organisms in these clades. First, species within the phylogenetic groups are mostly isolated from plants or fermented plant products and very rarely from animals (Mundt and Hammer 1968; Daeschel, Andersson and Fleming 1987). Second, the metabolic and physiological properties of the strains are reflective of a free-living lifestyle. Most species within these groups are aerotolerant by using a Mn (II) defense mechanism against oxygen toxicity (Daeschel, Andersson and Fleming 1987). Additionally, their optimal growth temperature is closer to temperatures of terrestrial and aquatic habitats as most species are able to grow at 15°C - some even grow at 2-4°C – but not at 45°C (Table 1.S1 Fig.1.3f). Third, they possess large genomes (Fig. 1.3a and b) encoding a versatile range of enzymes to utilize a wide spectrum of substrates, including pentoses, sucrose, lactose, mannitol, melezitose, cellobiose, nitrate, citric acid, and malic acid (Danner *et al.* 2003; Zheng *et al.* 2015a; Martino *et al.* 2016). Pentoses that are liberated upon degradation of plant materials as a result of hydrolysis of hemicellulose (Dewar, McDonald and Whittenbury 1963) are utilized by free-living lactobacilli through the pentose phosphate or phospoketolase pathways (Gänzle 2015). Interestingly, the ability to ferment pentoses is rarely found in yeast, suggesting a possible mechanism of niche partition between lactobacilli and yeast in their shared natural habitats (Mundt and Hammer 1968), which could be key to the success of lactobacilli in nature. Species that fit all three criteria well are *L. hokkaidonensis* and *buchneri* These species are isolated from grass silage, are aero-

tolerant, have a preference for pentose over hexose metabolism, an optimal growth temperature of 25 °C, and are psychrotrophic with a genome size of >2.3 kb (Tanizawa *et al.* 2015, Table 1.1)

1.4.2 Host-adapted lifestyle

The ability to colonize eukaryotic hosts benefits lactobacilli for several reasons; (*i*) their fastidious requirements for nutrients are satisfied in several host-associated niches; (*ii*) they often share the same food sources as the hosts (plants rich in simple and or complex carbohydrates); and (*iii*) they can use host animals as vectors to migrate to new habitats (Hammes and Hertel 2006; Mundt and Hammer 1968). Lactobacilli are found in vertebrates and insects. However, as described above, not all species isolated from animals are autochthonous, even those that differ markedly in the degree of specificity towards particular hosts or body sites, and the mechanisms by which symbiotic interrelationships are established and maintained. Examples are listed in Table 1, and below research on representative species that can serve as paradigms for host-associated lifestyles in lactobacilli are discussed.

1.4.2.1 Lactobacilli adapted to vertebrate hosts

Species that colonize vertebrate hosts cluster within the *L. delbrueckii, L. salivarius*, and *L. reuteri* groups, are monophyletic and predominantly comprise host-associated species. This suggests that the vertebrate-associated lifestyle is the outcome of a long-term evolutionary process that brought about a stable co-existence with vertebrate animals. However, lineages did not remain within specific host species, and the members of the phylogenetic groups differ in terms of host range, colonization site (gut, oral cavity, vagina), and the degree of specialization. This indicates that following initial adaptation to vertebrate hosts, further diversification and specialization occurred at the species level. Among the species for which the vertebrate lifestyle is best understood are *L. reuteri*, *L. ruminis*, *L. salivarius*, *L johnsonii, L. amylovorus*, and *L. iners* (Table 1). A number of characteristics reflect the adaptation of these species to gastrointestinal

environments. They tolerate bile acids, are highly acid-resistant, and ferment oligo- and polysaccharides present in the diet of their host species (Kakimoto *et al.* 1989; Grill *et al.* 2000; Lähteinen *et al.* 2010; Gänzle and Follador 2012; Ruiz, Margolles and Sánchez 2013; O' Donnell *et al.* 2015; Zheng *et al.* 2015a; Krumbeck *et al.* 2016). Additionally, these species grow optimally at 37°C and higher (Table 1.1), which reflects the body temperatures of most mammals and birds.

Vertebrate-associated lactobacilli typically colonize a range of host species. Exceptions include the human vaginal species *L. jensenii* and *L. iners*, and the pig-associated *L. amylovorus*. *L. amylovorus* is a dominant member of the porcine microbiota (Leser *et al.* 2002; Konstantinov *et al.* 2004, 2006; Chang *et al.* 2011; Kant *et al.* 2011) but is rarely detected in other animals (Nakamura 1981; Guan *et al.* 2003; Reti *et al.* 2013) suggesting that it is host-specific to pigs. The species dominates the microbiota on the *pars non-glandularis* region of the pig stomach, which is characterized by a dense biofilm composed of lactobacilli (Pedersen and Tannock 1989; Mann *et al.* 2014). In addition, *L. amylovorus* is one of few lactobacilli capable of utilizing amylose by the extracellular hydrolysis of starch (Gänzle and Follador 2012), a trait that is likely to contribute to the ecological fitness of the species in the distal intestinal tract of pigs (Regmi *et al.* 2011).

The highest degree of niche specialization in vertebrate-adapted lactobacilli occurs in the human vagina. The vaginal microbiota is dominated by *L. iners*, *L. crispatus*, *L. jensenii* and *L. gasseri* (Anderson *et al.* 2014; Mendes-Soares *et al.* 2014). *L. jensenii* and *L. iners* are only found in this niche and the latter species shows the highest degree of specialization observed among the currently known lactobacilli. Compared to other all other lactobacilli, *L. iners* has a smaller genome and more complex nutritional requirements, reflected by its inability to grow on standard growth media (Macklaim *et al.* 2011; Petrova *et al.* 2016). *L. iners* has apparently evolved to an almost obligate symbiotic lifestyle that is highly dependent on the human host. The presence of specific genes, such as the Fe-S protein cluster involved in defense against oxidative stress from H_2O_2 produced by other vaginal lactobacilli (Macklaim *et al.* 2011) also reflects specialization to the vaginal niche. Although biofilms are normally not observed in the healthy vagina, host

specificity of *L. iners* is likely achieved by specific adherence to epithelial cells in the vagina (Fig. 1. 4a; Macklaim *et al.* 2011).

The species *L. reuteri*, *L. ruminis*, *L. johnsonii*, *L. salivarius*, *L. cripatus*, *L. acidophilus*, and *L. vaginalis* have a broader host range and are found in different body sites (Table 1). However, the population structure of *L. reuteri*, *L. ruminis*, and *L. johnsonii* indicates that subpopulations within these species adapted and specialized to particular host animals. All three species separate in phylogenetic clusters that are highly reflective of host origin (Oh *et al.* 2010; Buhnik-Rosenblau *et al.* 2012; O' Donnell *et al.* 2015). For *L. reuteri*, these clusters have been established by Amplified Fragment Length Polymorphism, Multilocus Sequence Analysis (Oh *et al.* 2010 Fig. 1.5a), and whole genome phylogenies (Wegmann *et al.* 2015; Duar *et al.* 2017). The genome content of strains from different phylogenetic clusters is reflective of the niche characteristics in respective hosts (Frese *et al.* 2011). *L. reuteri* is regarded as autochthonous to the human gut (Reuter 2001) and has been found to be a prevalent member of the microbiota of traditional agriculturalist societies (Martínez *et al.* 2015). The genomes of human strains of *L. reuteri* are characterized by a closed pangenome and extensive deletion of large, adhesin-like surface proteins, but the ability to utilize glycerol and propanediol as electron acceptors, suggesting growth in the intestinal lumen (Frese *et al.* 2011; Walter, Britton and Roos 2011). In contrast, rodent *L. reuteri* strains possess several large-adhesin like surface proteins and colonize by adhering to the surface of the squamous stratified epithelia of the forestomach of mice on which they form biofilms (Walter *et al.* 2005, 2007; Frese *et al.* 2013, Fig. 1.5a). Host specificity in *L. reuteri* has been experimentally demonstrated in competition experiments in gnotobiotic mice and more recently in chickens (Oh *et al.* 2010; Frese *et al.* 2011; Duar *et al.* 2017). *L. reuteri* isolated from both rats and mice cluster together and rat isolates are very competitive in mice. Similarly, isolates from chicken and turkeys group in the same phylogenetic lineages (Oh *et al.* 2010; Frese *et al.* 2011; Duar *et al.* 2017)

Table 1.1- Genomic and metabolic characteristics of species representing the different lifestyles of lactobacilli

Group	Organism	Habitat	OT[a] (°C)	Genome size (Mb)	GC (%)	Lifestyle-associated traits	Mechanisms of host specificity	References
Free-living								
vac	*L. hokkaidonensis*	Grass/silage	25	2.3	38.1	pentose fermentation, aerotolerance	N/A	Tohno *et al* .(2013), Tanizawa *et al.* (2015)
buc	*L. buchneri*	Grass/silage	37	2.5	44.4	pentose fermentation, plant cell wall degradation	N/A	Heinl *et al.* (2012) *Kleinschmit et al.* (2006)
Nomadic								
pla	*L. plantarum*	Fruit flies; vertebrate digestive tract; plants and dairy products	37	3.2	44.5	bile resistance; metabolic versatility; two component systems.; extracellular proteins	N/A	Martino *et al.* (2016); Siezen *et al* (2010)
cas	*L. casei*	raw and fermented dairy; silage, fermented vegetables, vertebrate digestive tract	30	2.8	46.5	metabolic flexibility; adhesion to intestinal villi; bile resistance; environmental sensing and adjustment; prototrophic to most amino acids	N/A	Cai et al (2007, 2009); Broadbent *et al.* (2012)
cas	*L. rhamnosus*	raw and fermented dairy, oral cavity, digestive tract of vertebrates, vagina	37	2.9	46.7	metabolic flexibility, fermentation of a wide range of carbohydrates; bile resistance; pili-mediated mucus adhesion; immunomodulation.	N/A	Douillard (2013,2013a); Ceapa (2015,2016);
Vertebrate-adapted								
sav	*L. ruminus*	Digestive tract; predominant in the bovine rumen; reported in humans, dogs, pigs, cats horses and primates.	37	2.1	43.5	bile and acid resistance; motility, substrate foraging; immunomodulation	Unknown	O'Donnell *et al*. (2015); Forde *et al.* (2011)
reu	*L. reuteri*	Proximal digestive tract; prevalent in rodents, pigs and chickens; reported in humans, dogs, minks, lambs, giraffes, cats and horses	37	1.9	38.6	bile and acid resistance; adhesion and biofilm formation	Epithelial adherence	Oh *et al* (2011); Frese *et al***.** (2013)

del	*L. amylovorus*	Digestive tract; prevalent in swine; reported in chickens and horses.	37	2.0	37.8	bile and acid resistance; extracellular amylases, surface-attached "S-layers"; immunomodulation	Unknown	Kant *et al.* (2011); Grill *et al*. (2001)
sav	*L. salivarius*	Human oral cavity and digestive tract.; reported in breast milk and vagina and feces of pigs, raccoons, chickens and hamsters	37	2.0	32.5	bile resistance, bacteriocin production (Megaplasmid encoded)	N/A	Raftis *et at.* (2011, 2014); Li *et a.l* (2007)
del	*L. johnsonii*	Proximal digestive tract of rodents and poultry	37	1.8	34.5	Bacteriocin production and bile resistance	Unknown	Buhnik-Rosenblau *et al.* (2012); Pridmore (2004)
del	*L. iners*	Human vagina	37	1.3	32.5	Fe-S - defense against peroxide. Glycogen fermentation, adhesion	Epithelial adherence	Petrova *et al*. (2016); Macklaim *et al* (2011)
Insect-adapted								
del	*L. apis*	Bee	37	1.7	36.6	biofilm formation in the hindgut	Adherence/ Biofilm	Ellegaard *et al*. (2015); Anderson *et al.* (2013)
mel	*L. mellis*	Bee	30	1.8	36.2	putative exopolysaccharide formation, niche partition with other members of bee core microbiota	unknown	Ellagaard *et al*. (2015); Corby-Harris *et al*. (2014)
kun	*L. kunkeei*	Flowers, grapes, bees	30	1.5	36.4	fructophilic, resistant to phenolics and honey-desiccation	N/A	Vojvodic *et al*.(2013), Anderson *et al*. (2013), Endo *et al*. (2013) Maeno *et al* (2016)

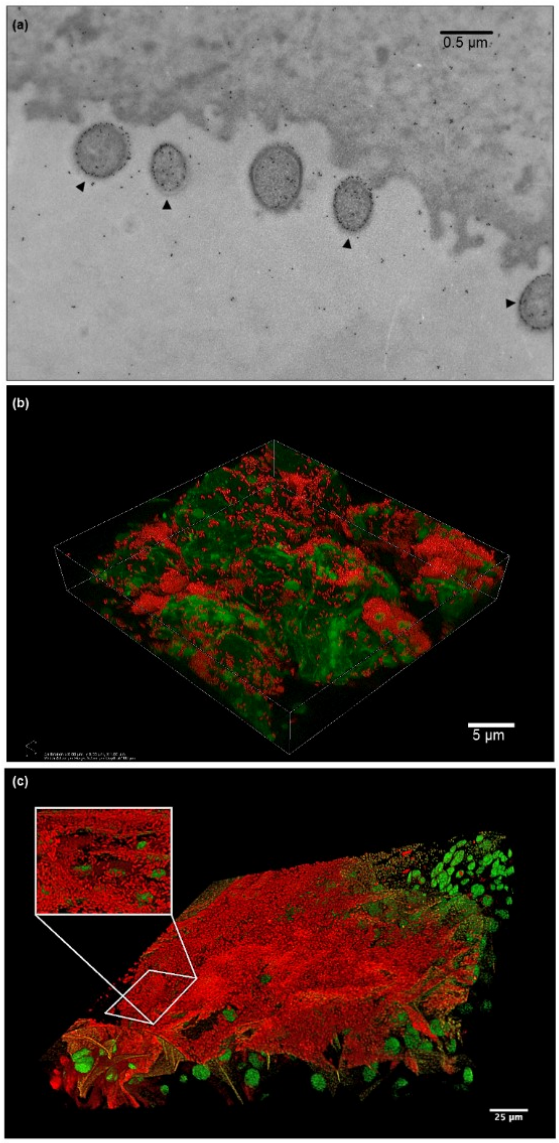


Figure 1.4 Association of lactobacilli with host epithelia.
(a) Transmission electron micrograph image of immunogold- labeled *L. iners* cells in association with human vaginal epithelial cells, with *L. iners* cells indicated with an arrow (image from Macklaim *et al.* 2011). (b) Three dimensional confocal micrograph taken 24 hours after colonizing a germ-free mouse with a pure culture of the rat isolate *L. reuteri* 100-23. The specimen were stained with propidium iodide and imaged by confocal microscopy, which results in the bacterial cells to be colored red and the forestomach epithelium to appear green, as described by Frese *et al*. (2013). The image was taken by Christian Elowsky and Steven Frese at the University of Nebraska at Lincoln Microscopy Core. (c) Biofilm (red) composed of Lactic Acid Bacteria attached to a honeybee's crop (green)(Vásquez *et al.* 2012). Images used under the Creative Commons Attribution (CC BY) license.

These findings demonstrate that *L. reuteri* has adapted to groups of related host species such as rodents or poultry that possess similar niches in their intestinal tracts and whose social behavior allows horizontal transfer of bacteria (Oh *et al.* 2010).

L. reuteri has been established as a model species to study mechanisms of host adaptation in lactobacilli (Walter, Britton and Roos 2011; Kwong and Moran 2015). Functional studies with loss-of-function mutations have demonstrated that the ecological success of rodent strains in the forestomach depends on biofilm formation (Fig. 1.4b), which is only observed in rodent strains, and resistace to gastric acidity (Walter *et al.* 2007; Frese *et al.* 2013; Krumbeck *et al.* 2016). Inactivation of one single serine-rich surface adhesin specific to rodent strains with a devoted transport system (the SecA2-SecY2 pathway) completely abrogated biofilm formation, indicating that initial adhesion represented the most significant mechanism underlying host-specific colonization (Frese *et al.* 2013). Similar mechanistic studies are lacking in other species of lactobacilli but comparable genomic patterns of host adaptation are observed, e.g. for *L. ruminis*. Human isolates of *L. ruminis* are aflagellate and non-motile while bovine, equine and porcine isolates are motile, with the latter two being hyper-flagellated (O' Donnell *et al.* 2015). These differences in the expression of flagella and motility could reflect adaptation to the conditions in different hosts. Overall, the data available for *L. reuteri* and *L. ruminis* indicate that some lactobacilli evolved to a high degree of host-specialization. Moreover, robust clustering in defined phylogenetic groups based on host origin indicates that these host associations are maintained over evolutionary timescales. Finally, the high fidelity in epithelial recognition for biofilm formation of bacterial strains, as demonstrated for *L. reuteri* (Frese *et al.* 2013), provides a mechanism by which lineages are reliably transmitted from generation to generation and maintained over both ecological and evolutionary time scales.

Other host-adapted species appear to have a less specific and more 'promiscuous' lifestyle, both in terms of host range and body site. *L. salivarius* is indigenous to the human oral cavity (Rogosa *et al.* 1953) and is one of few *Lactobacillus* species that has been consistently recovered

from the feces of human individuals for at least 18 months (Tannock *et al.* 2000). *L. salivarius* has also been obtained from breast milk (Martín *et al.* 2006) and a variety of body sites including the intestinal mucosa (Molin *et al.* 1993), tongue, rectum (Ahrné *et al.* 1998) and the vagina (Vera Pingitore *et al.* 2009). This species is also found in pigs (Mackenzie *et al.* 2014), chicken (Hammons *et al.* 2010) hamsters (Rogosa *et al.* 1953), and horses (Yuki *et al.* 2000). Phylogenetic analysis of strains from a variety of sources did not show clustering by origin. However, many isolates show signs of ongoing adaptation by genome decay (Raftis *et al.* 2011). *L. vaginalis* and *L. gasseri* can be detected in oral and fecal microbiota of the same species (Dal Bello and Hertel 2006) and they are also members of the vaginal microbiota. Therefore, it appears that these species maintain more dynamic and flexible lifestyles regarding host range and ecological niche in comparison to *L. reuteri* and *L. ruminis*.

1.4.2.2 Lactobacilli associated with invertebrate hosts.

The association of lactobacilli with invertebrates as abundant members of the microbiome is a recent discovery (Shrivastava 1982; Engel and Moran 2013). Insect-associated species are distributed across the *Lactobacillus* phylogeny (McFrederick *et al.* 2012) (Fig 1.2) and cluster in phylogenetic groups with different levels of host specificity. Species associated with bees cluster in the *L. kunkeei* and *L. mellifer* groups and in the *L. helsinborgensis* clade of the *L. delbrueckii* group (Fig 1.2), which were termed as the Firm 4 and Firm 5 phylotypes prior to description of the species (Ellegaard *et al.* 2015). This finding suggests that association with the bee gut occurred in independent events (events 6 and 4, Fig. 1.2). Species of the *L. fructivorans* group (Fig. 1.2) are also often associated with bees but appear to be between species by floral transmission (McFrederick *et al.* 2012).

Species belonging to all four groups are characterized by having small genome sizes (Zheng *et al.* 2015a; Maeno *et al.* 2016, Fig.1.2) and extremely limited carbohydrate fermentation capabilities (Ellegaard *et al.* 2015), being essentially restricted to a “sucrose and maltose diet”.

Heterofermentative lactobacilli associated with bees are fructophilic; they lack alcohol dehydrogenase activity and depend on the availability of fructose as electron acceptor (Endo, Futagawa-Endo and Dicks 2009; Filannino *et al.* 2016; Maeno *et al.* 2016). Bee-associated lactobacilli in the homofermentative *L. mellifer* group share the restricted carbohydrate fermentation ability (Zheng *et al.* 2015a). These patterns of carbohydrate restriction are vastly different from vertebrate-adapted lactobacilli, which retain the ability to degrade a wider variety of carbohydrates despite their small genome size. It is likely that these differences reflect adaptations not only to the host's diet (i.e. honey, nectar and pollen for bees) but also the differences in the competitive interactions that occur within the gut environments. Compared to vertebrates, bees harbor relative simple microbial communities composed of 9 bacterial species clusters, and there is compelling evidence that species occupy distinct and complementary metabolic niches within the bee gut (Powell *et al.* 2016).Therefore, specialization as a means of niche partitioning and syntrophic interaction seem to be one of the key mechanisms to the ecological success of bee-associated lactobacilli species (Kwong and Moran 2016).

Lactobacillus species are often dominant members of the microbiota of some species of Hymenoptera (ants, bees, and wasps) (Kwong and Moran 2016). However, only honey and bumble bees have been described to date to harbor selective lineages of lactobacilli, suggesting a high degree of host-specificity in these hymenopteran hosts (McFrederick *et al.* 2013). Both the *L. mellifer* group and *L. helsinborgensis* clade are almost ubiquitously represented in individual bees and are particularly abundant in adult workers and the queen bee, and individual lineages can be specific to honey and bumble bees (Vásquez *et al.* 2012; Kwong and Moran 2016). These species are oxygen-sensitive and have not been found outside the bee gut, and are likely obligate symbionts colonizing the anoxic regions of the distal hindgut. Genomic signatures of these species are in agreement with those of adapted symbionts (Lo, Huang and Kuo 2016). All species have small genomes, (< 2.1 Mb) with low GC contents ranging from 34.6 to a 36.6%. Most strains can grow at 15 °C and optimally at temperatures significantly lower than those adapted to

vertebrates (Fig 1.3e, Table 1.S1). The presence of genes involved in the utilization of trehalose; a disaccharide that is used for energy storage in insects, also emphasizes their adaptation to the insect gut (Ellegaard *et al.* 2015). Consistent with the adaptation to a sugar-rich environment, species of the *L. helsinborgensis* clade harbor a large number of PTS systems, carbohydrate transporters and a variety of modification enzymes including glucosidases, hydrolases, isomerases, racemases, epimerases, aldolases; more than most lactobacilli. Moreover, *L mellifer* and *L. mellis* encode strain specific genes with putative function in exopolysaccharide biosynthesis presumably involved in biofilm formation (Ellegaard *et al.* 2015).

Contrary to homofermentative bee-associated lactobacilli*,* the *L. kunkeei* group are dominant members in the crop microbiota of bee but can also be detected in pollen, nectar and hive materials, as well as from fresh flowers and fruits (Endo *et al.* 2012; Neveling, Endo and Dicks 2012; Anderson *et al.* 2013). *L. kunkeei* migrates frequently between honey bees and stingless bees and shows no evidence of specificity to either host, suggesting that the species is more 'promiscuous' than the members of the *L. mellifer* group and *L. helsinborgensis* clade (Tamarit *et al.* 2015). However, the genomic features and metabolic and biochemical traits of *L. kunkeei* clearly reflect adaptations to the bee gut and the hive environment (Tamarit *et al.* 2015). The phylogenetic group has small genomes (< 1.5 Mb), reduced GC content and grows at 15 °C (Fig.1. 3, Table 1.1). *L. kunkeei* is obligately fructophilic*,* osmotolerant and resistant to high concentration of phenolic acids present in pollen. Phenolic acids are used as electron acceptors (Filannino *et al.* 2016). Moreover, it resists the antimicrobial activity of royal jelly (Vojvodic *et al.* 2013) and the desiccant conditions in honey (Endo *et al.* 2012; Vojvodic *et al.* 2013). *L. kunkeei* is a major component of the biofilm that is found in the bee crop as determined by 16S rRNA sequencing (Vásquez *et al.* 2012) (Fig. 1.4c). The ability of *L. kunkeei* to tolerate aerobic conditions is consistent with its capacity to migrate between hosts and the environment, such as fruits and flowers. However, the exact role of the environmental niches in the lifestyle of *L. kunkeei* is unclear. The species might be able to stably colonize fruits and flowers and/or uses them for

transmission. Alternatively, *L. kunkeei* might just be allochtnonous to fruits and flowers that gets deposited at these sites during bee pollination and foraging (McFrederick *et al.* 2012; Tamarit *et al.* 2015).

Species in the *L. fructivorans* group are shared between plants and insects including sweat bees and *Drosophila*. From its six species two were isolated from insects (*L. fructivorans*, *L. vespulae*), two from flowers (*L. ixorae*, *L. florum*), and two from fermented food products and therefore have an unknown environmental niche (*L. sanfranciscensis* and *L. homohiochi*) (Kitara, Kaneko and Goto 1957; Endo *et al.* 2010; Vogel *et al.* 2011; Wong, Ng and Douglas 2011; McFrederick *et al.* 2013; Techo *et al.* 2016, Fig. 1.S1). Although their lifestyle has not yet been studied, their reduced genome size (<1.5 Mb, with *L. sanfransciscensis* possessing one of the smallest genomes of all lactobacilli) and GC content (Fig. 1.3a-d) show the classic hallmarks of symbionts (Lo, Huang and Kuo 2016). In addition, species are either anaerobic or microaerophilic, reflecting adaptation to oxygen limiting conditions, which are likely to be found in the guts of insects but not in the environment (Pot *et al.* 2014, Table S1). We have classified this group as 'insect-associated' but more research is needed to elucidate the exact lifestyle of its members.

Contrary to bees, *Drosophila* species do not harbor a defined core-microbiota and the composition varies widely between wild and lab species. In fact, the microbiota of fruit flies is composed mainly of *Acetobacte*r and *Lactobacillus* species found in other habitats, including the environment, dairy and vertebrate animals (Chandler *et al.* 2011; Wong, Ng and Douglas 2011; Erkosar *et al.* 2013; Wong, Chaston and Douglas 2013). In addition, diet plays a major role in shaping the microbiome of *Drosophila* spp. (Wong *et al.* 2015). It appears therefore, that the association of lactobacilli with fruit flies is less host-restricted, more dynamic and includes the immediate environment of the insects (Wong *et al.* 2015). Such a lifestyle can be considered 'nomadic'.

1.4.2.3 “Nomadic” species of lactobacilli

Most of the *Lactobacillus* species found in the human gut do not form stable populations and have been described as allochthonous as they are derived from food or feed (Tannock et al. 2000; Walter et al. 2001; Tannock 2004; Walter 2008). However, although not autochthonous in the classical sense, some *Lactobacillus* species, such as *L. plantarum*, *L. casei*, *L.paracasei*, and *L. rhamnosus* possess adaptations to gut ecosystems and the oral cavity that allow them to persist for at least a limited time (Table 1.1). These species possess large genomes with little evidence for specialization to particular habitats, and they are found in vertebrate and invertebrate hosts and different body parts (gut, oral cavity, vagina), and in food materials, such as meat, fish, vegetables and raw or fermented dairy products (Kandler 1986; Stiles and Holzapfel 1997; Heilig *et al.* 2002; Wall *et al.* 2007; Delgado, Suárez and Mayo 2010; Siezen *et al.* 2010; Ceapa *et al.* 2016; Rossi *et al.* 2016). Recent research has provided convincing evidence that they represent examples of a nomadic lifestyle (Martino *et al.* 2016)

Nomadic *Lactobacillus* species cluster in two phylogenetic lineages, the *L. plantarum* group and a cluster within the *L. casei* group (Fig. 1.2). Their large genomes correspond to increased metabolic flexibility. Comparable to free-living lactobacilli, *L. plantarum* and *L. casei* retained the capacity for conditional respiration (Brooijmans, de Vos and Hugenholtz 2009; Zotta et al. 2016). In addition, *L. plantarum* WCFS1 encodes a large spectrum of sugar uptake and utilization cassettes, allowing the organism to grow on numerous carbon sources. The genome of *L. plantarum* WCFS1 also encodes over 200 putative extracellular proteins, most of which are displayed in the cell surface. The presence of such proteins in plant-associated bacteria is often related to the degradation and utilization of plant oligo and polysaccharides, and likely contributes to the flexibility of *L. plantarum* to interact with its environment in different habitats (Siezen and van Hylckama Vlieg 2011). *L. casei* ATCC393, a strain isolated from cheese, is capable of synthetizing most amino acids (except for valine, leucine and isoleucine), and is thus able to thrive in protein-limited environments. It utilizes a great variety of carbohydrates (Cai *et al.* 2007) and

contains 16 two-components systems; the highest number observed among lactobacilli, indicating that gene expression is adjusted to diverse environments (Cai *et al.* 2009). The diversity of these three species has been extensively studied by both phenotypic and genotypic approaches (Bringel, Curk and Hubert 1996; Torriani *et al.* 2001; De Las Rivas *et al.* 2005; Molenaar *et al.* 2005; Cai *et al.* 2007, 2009; Diancourt *et al.* 2007; Siezen *et al.* 2010; Broadbent *et al.* 2012; Smokvina *et al.* 2013a; Martino *et al.* 2016). These approaches demonstrated high genetic and phenotypic intra-species diversity. Comparative genomic analysis of 54 *L. plantarum* strains demonstrated the absence of environmental specialization (Martino *et al.* 2016, and Fig. 1.5b), which had been already hypothesized in previous studies (Molenaar *et al.* 2005; Siezen *et al.* 2010). Genes involved in exopolysaccharide biosynthesis, sugar metabolism and the secretome showed the most variability amongst strains but did not relate to specialization to any specific habitats (Martino *et al.* 2016). Similarly, *L. paracasei* and *L. casei* did not show a correlation between the habitat and phylogenetic position as determined by core and pan-genome phylogenies coupled with analyses of variable regions (Cai *et al.* 2009; Smokvina *et al.* 2013a). *L. rhamnosus* has been isolated from a large variety of habitats, including the human gastrointestinal tract, vaginal cavity, oral cavity and cheese, exemplifying its remarkable ecological adaptability. Comparable to *L. plantarum* and *L. casei*, *L. rhamnosus* likely resides in multiple niches, illustrating its nomadic lifestyle (Douillard *et al.* 2013a).

L. plantarum, *L. casei*, and *L. rhamnosus* do not form stable population in animal hosts but possess adaptive features to niches associated with humans and animals that contribute to their persistence (Walter 2008). For example, *L. plantarum* shows high tolerance to gastric juice and bile acids (Bron *et al.* 2004b; van den Nieuwboer *et al.* 2016). *L. casei* adheres to intestinal villi (Galdeano and Perdigón 2004) and both *L. casei* and *L. paracasei* resist bile (Alcántara and Zúñiga 2012) (Wang *et al.* 2010). *L. rhamnosus* possesses mucus-binding pili that might interact with the host epithelia in the oral cavity and the small intestine (Kankainen *et al.* 2009). *L. plantarum* responds to the gastrointestinal environment of mice by regulating a large array of

genes (Bron *et al.* 2004a). Interestingly, persistence of *L. plantarum* in the gastrointestinal tract of mice increases after only three passages (van Bokhorst-van de Veen *et al.* 2013). These studies suggest that some *Lactobacillus* species can adapt to intestinal ecosystems and temporarily persist despite not being autochthonous members of the resident microbiota.

Taken together, the findings indicate that some *Lactobacillus* species have evolved a nomadic lifestyle that exerts diverse selective pressures rather than promoting niche specialization. Genomic and phenotypic characteristics of strains of these species appear unrelated to the origin of isolation, which highlights their ability to migrate across environments; in line with their ubiquitous presence and their ability to thrive on various substrates. This feature can be also seen as a strategy of dissemination, or from an ecological perspective, dispersal (Vellend 2010). During evolution, these species, originally associated with plants, may have developed the ability to inhabit the gut of animals feeding on plants, favoring dissemination to new habitats. Dispersal influences the dynamics, composition and structure of communities and the distribution and abundance of species. From an evolutionary perspective, it affects processes such as local adaptation, speciation and the evolution of traits that ultimately impact the natural history of species (Dieckmann, O'Hara and Weisser 1999). Nomadic *Lactobacillus* species could have evolved dispersal traits in the form of colonization factors of host animals, which allow these immotile bacterial species to disseminate. Nomadic lifestyles of lactobacilli have also been identified in insects such as some species of Hymenoptera (sweat bees and ants) and fruitflies, (McFrederick *et al.* 2013; Matos and Leulier 2014) which represent excellent vectors for dissemination for bacteria that have their main habitat in plants and fruits. However, *Lactobacillus* lifecycle might even be more complex and dynamic, beginning with intestinal waste, followed by mechanical distribution to and among plants, and return to the host via the oral and alimentary cavity, as suggested in 1968 by Mundt and Hammer. Further studies should be directed to reconstructing the natural and evolutionary history of nomadic lactobacilli in both vertebrates and

invertebrates order to better understand their adaptation process and the relative importance of free-living and host associated niches.

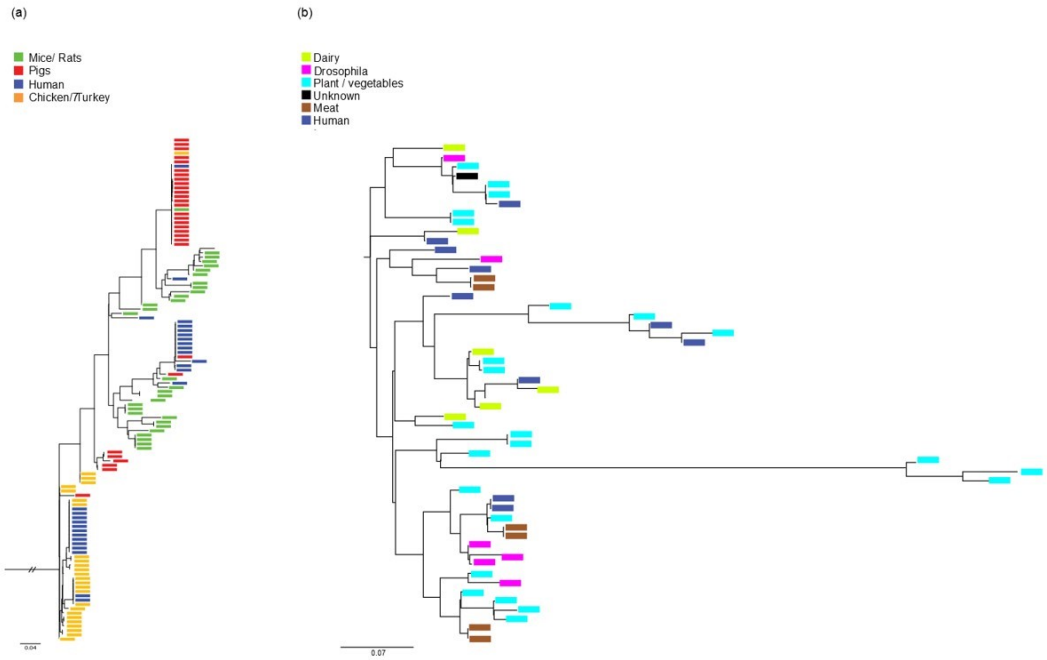


Figure 1.5- Maximum likelihood trees comparing the phylogenetic structure of the (a) host-adapted species *L. reuteri* and the (b) nomadic *L. plantarum*

Tips of the branches are color coded by the strains' origin of isolation. The phylogeny of *L. reuteri* tree was inferred by multilocus sequencing analysis of 116 strains as described by Oh *et al.* (2010)

1.5 A model for the evolution of lifestyle transitions in the *Lactobacillus sensu lato*

The synthesis of phylogenomic, metabolic, and data presented in this review provides a highly consistent view on the evolution of distinct lifestyles of lactobacilli (Fig. 1.6). A free-living ancestry

for the *Lactobacillus sensu lato* is logical as symbioses with plants have not been described and the diversification from the bacilli predates the emergence of animals. From the ancestral state, the group has diversified and evolved lifestyles that cover the entire spectrum from free-living to strictly host-adapted, with a substantial variation in the reliance on environmental niches and the degree of host-specificity.

The phylogenomic data supports a model by which *Lactobacillus* lineages have diversified and evolved symbiotic lifestyles on five separate occasions (event 1-5 in Fig. 1.2), resulting in the *L. delbrueckii, L. salivarius*, *L. reuteri*, *L. mellifer*, and *L. kunkeei*/*L. fructivorans* phylogenetic groups. This evolutionary process is reflected by adaptations to the host environment (bile and acid tolerance, growth at host body temperature, metabolic adaptations to insects) and genomic patterns (genome decay, decreased GC content, loss of biosynthetic enzymes) consistent with those found in other bacterial symbionts (Lo, Huang and Kuo 2016). Host-adapted lactobacilli differ in the degree of niche specialization and host dependence, and lifestyles can range from 'promiscuous' to completely host restricted, with *L. iners* representing the most extreme cultural representative. Selective epithelial adhesion (often followed by the formation of biofilms) appear to be a key mechanism by which lactobacilli maintain stable associations with hosts over evolutionary times as most animal sites with highly adapted species are characterized by adherent cells, e.g. the vagina, the crop of insects and birds, the forestomach of rodents and horses, and the pars esophagus in pigs (Fuller and Brooker 1974; Pedersen and Tannock 1989; Tannock 1992; Yuki *et al.* 2000; Vásquez *et al.* 2012; Frese *et al.* 2013; Mann *et al.* 2014).

These host-adapted lifestyles likely evolved after ancestral plant, fruit, and flower associated lactobacilli became exposed to animals that were feeding on their primary habitats. Although this exposure was initiallly coincidental, bacterial traits that allowed the bacteria to tolerate the conditions in the host and allowed temporal persistence contributed to the transmission and hence, dispersal of lactobacilli. As such traits would ultimately increase the success of the lineages in their primary habitats, they could be shaped by natural selection even if they did not

allow stable colonization of the host, gradually increasing the relevance of host niches for the overall lifestyle. The result was the evolution of distinct and dynamic lifestyles that differ in the degree by which the microbes rely on environmental and host niches, and their dynamic interactions. Such 'nomadic' lifestyles remain represented within the genus *Lactobacillus* and might well constitute a transitional state from the free-living lifestyle to a specialized symbiosis.

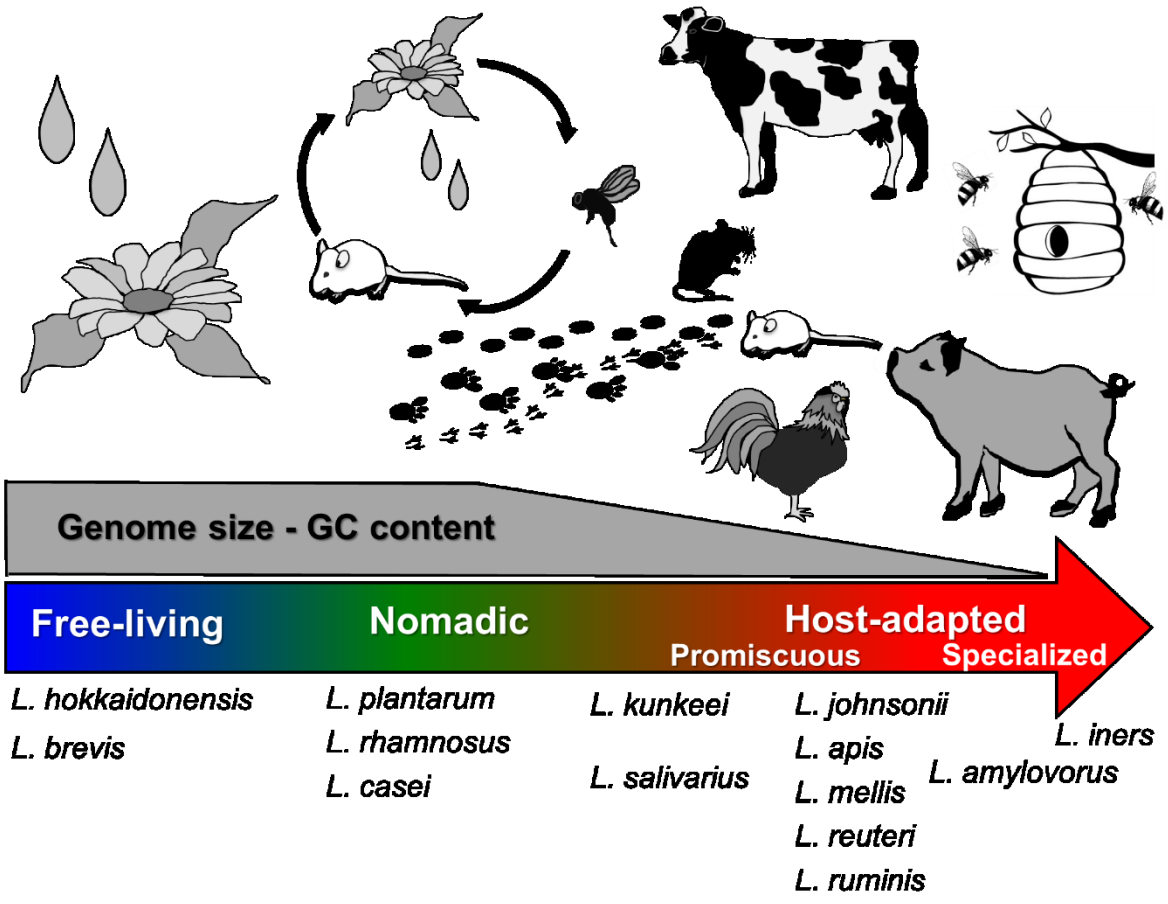


Figure 1.6 Model of the evolution of lifestyles in the genus *Lactobacillus*

Lifestyle evolution of lactobacilli from free-living to strictly host-adapted species. Representative species discussed in the text are included according to their lifestyle and their reliance on environmental niches and the degree of host-specificity.

1.6 Open questions

In symbiotic associations, both host and symbiont can reciprocally affect each other's evolution (Moran 2006). When stably associated with a host, the bacterial symbiont is likely to adapt, which can lead to specialization and host-restriction. The ultimate result is an obligate symbiosis, in which the microbe depends on the host for survival. *L. iners* represents *a* classic example of such evolutionary process (Macklaim et al. 2011; Petrova et al. 2016). Rodent lineages of *L. reuteri,* although not yet obligate symbionts, have specialized to a degree that restricts their host range. However, so far it had remained unclear whether non-rodent lineages of *L. reuteri* have evolved to become host adapted, and open questions remain about the evolutionary relationships within lineage VI, which is shared by human and poultry isolates. **Chapter two** describes a study that tested host adaptations of *L. reuteri* strains to swine, poultry and human volunteers.

Host anatomical sites where most host-adapted species colonize (bee cop, mouse forestomach and human vagina) have the characteristic of being lined by a stratified squamous tissue that allows lactobacilli to adhere and form biofilms (Fig. 1.4). These epithelia might therefore constitute an anatomical feature that evolved in the host to facilitate specific colonization of the beneficial symbionts. In mice, biofilm formation, in the forestomach is restricted to rodent strains of *L. reuteri (Frese et al. 2013).* In order to maintain such specificity, rigorous environmental sensing and selective epithelium recognition are likely to play a crucial role. Biofilm formation is often orchestrated in response to external signals recognized by signal transduction systems. Previously, a two component system (TCS) 70529-30 was identified to be specific to rodent strains, but its relevance in biofilm formation had remained unexplored (Frese *et al.* 2011). Isogenic deletion mutants of the histidine kinase (*hk70529*) and the response regulator (*rr70530*) were generated in the rodent strain *L. reuteri* 100-23 and their ability to produce biofilms was

determined in monoassociated mice and further characterized using *in vitro* techniques. Findings are presented in **Chapter three**

Genes involved in adherence and biofilms formation are absent in most human strains of *L. reuteri* (Frese *et al.* 2011) thus suggesting a planktonic lifestyle for *L reuteri* in the human gastrointestinal tract. (Frese *et al.* 2011; Walter, Britton and Roos 2011). While lacking the genetic machinery to form biofilms, human strains conserve a 58-gene *pdu-cbi-cob-hem* cluster encoding functions in reuterin production, cobalamin biosynthesis, and glycerol and 1,2 propanediol (1,2 PD) utilization. Deoxy hexoses (fucose and rhamnose) derived from dietary and endogenous glycans are converted into 1,2 PD by a number of gut bacteria, making 1,2 PD readily available in the human gastrointestinal tract. It has been hypothesized that utilization of 1,2 PD might be a relevant colonization factor for *L. reuteri* in the human gut. **Chapter four** explores the ecological relevance of the *pdu-cbi-cob-hem* cluste*r* in the context of cross-feeding of 1,2 PD produced from the fermentation of fucose and rhamnose by two different gut bacteria.

Findings from probiotic research suggest that host-specificity might not a be requisite for lactobacilli to provide beneficial effects upon the host. The list of *Lactobacillus* species with recognized probiotic properties comprises host-adapted species such as *L. reuteri*, *L. johnsonii* and *L. acidophilus*, as well as nomadic and free-living organisms such as *L. plantarum, L. casei* and *L. fermentum* (Floch *et al.* 2015). This indicates that lactobacilli, irrespective of lifestyle, can be introduced into the gastrointestinal tract and remain viable. Nonetheless, is it a logical working hypothesis that those adapted to gastrointestinal environments will remain physiologically and are more likely to influence host physiology. Work in **Chapter 5** presents a unique approach to exploit the metabolic capacity of lactobacilli adapted to the vertebrate gut for therapeutic purposes. Strains of the host-adapted species *L. ruminis, L. johnsonii, L. amylovorus, and L. salivarius*

isolated from the porcine gut, are characterized by their ability to degrade meta-stable peptides involved in the pathogenesis of celiac disease.

Finally, conclusions from research presented in this thesis and the implications for future studies are presented in **Chapter 6.**

1.7 Supplementary material

Table S1.1 Metadata of type strains with lifestyle assignment in Fig. 1.2

Group	Species	Optimal growth temperature (°C)	Growth at 15 °C	Growth at 45 °C	Atmosphere	Genome size (Mb)	GC content (%)	Type Strain	CDSs	Genome Accession No.	Main Habitat	Source of Isolation
	Free-living											
bre	*L. brevis*	30	Y	N	aerobic	2.5	45.9	ATCC 14869	2291	NZ_AWVK00000000.1	food/environmen	human faeces
bre	*L. hammesii*	30	Y	N	microaerophilic	2.8	49.4	DSM 16381	2506	NZ_AZFS00000000.1	plants/vegetables	wheat sourdough
bre	*L. koreensis*	30	Y	N	facultative anaerobi	3.0	49.2	JCM 16448	2608	NZ_CP012033.1	plants/vegetables	Kimchi
bre	*L. namurensis*	30	Y	N	microaerophilic	2.5	52.0	DSM 19117	2162	NZ_AZDT00000000.1	plants/vegetables	sourdough, manufactured with wheat, rye and spelt flour
bre	*L. parabrevis*	37	Y	N	microaerophilic	2.6	49.0	ATCC 53295	2314	NZ_ARTH00000000.1	fermented vegetable	senmaizuke
bre	*L. paucivorans*	28	Y	N	anerobic	2.4	49.1	DSM 22467	2129	NZ_JQCA00000000.1	fermented cereal	brewery
bre	*L. senmaizukei*	30	Y	N	microaerophilic-ana	2.2	48.6	DSM 21775	2064	NZ_AYZH00000000.1	fermented vegetable	pickles
bre	*L. spicheri*	30	Y	N	microaerophilic	2.7	55.9	DSM 15429	2410	NZ_AZFC00000000.1	fermented cereal	rice sourdough
bre	*L. zymae*	30	Y	N	microaerophilic	2.7	53.6	DSM 19395	2392	NZ_AZDW00000000.1	fermented cereal	artisanal wheat sourdough
buc	*L. buchneri*	37	Y	N	facultative anaerobi	2.5	44.4	DSM 20057	2288	NC_018610.1	fruits	tomato pulp
buc	*L. diolivorans*	30				3.3	40.0	DSM 14421	2906	NZ_AZEY00000000.1	silage	maize silage
buc	*L. fanaganis*	30	Y	Y	microaerophilic	2.8	42.1	DSM 18382	2204	NZ_BAKI00000000.1	fermented cereal	composting material, of distilled shochu residue
buc	*L. hilgardii*	30	Y	N	facultative anaerobi	2.6	39.6	DSM 20176	2336	NZ_ACGP00000000.1	fermented fruits	wine
buc	*L. kisonensis*	30	Y (growth at 10	N	microaerophilic	3.0	41.7	DSM 19906	2647	NZ_AZEB00000000.1	fermented vegetable	non-salted pickle solution used in production of sunki (Japanese traditional, fermented leaves and stems of red turnips)
buc	*L. otakiensis*	30	Y (growth at 10 °C)	N	microaerophilic	2.3	42.4	DSM 19908	2204	NZ_AZED00000000.1	fermented vegetable	non-salted pickle solution used in production of sunki (Japanese traditional, fermented leaves and stems of red turnips)
buc	*L. parabuchneri*	28	Y		facultative anaerobi	2.6	43.4	DSM 5707	2311	NZ_AZGK00000000.1	human saliva	human saliva
buc	*L. parafarraginis*	30	N	N	microaerophilic	3.1	45.2	DSM 18390	2232	NZ_BBAR00000000.1	fermented cereal	composting material, of distilled shochun residue
buc	*L. rapi*	30	Y (growth at 10	N	microaerophilic	2.9	43.0	DSM 19907	2550	NZ_AZEI00000000.1	fermented vegetable	non-salted pickle solution used in production of sunki (Japanese traditional, fermented leaves and stems of red turnips)
buc	*L. sunkii*	30	Y (growth at 10	N	microaerophilic	2.7	42.0	DSM 19904	2488	NZ_AZEA00000000.1	fermented vegetable	non-salted pickle solution used in production of sunki (Japanese traditional, fermented leaves and stems of red turnips)
buc	*L. kefiri*	30	Y	N		2.7	41.7	DSM 20587	2007	NZ_AYVO00000000.1	fermented food	kefir grains
buc	*L. parakefiri*	30	Y	N	facultative anaerobi	4.9	41.6	DSM 10551	4704	AZNO00000000.1	fermented food	fermented grains
col	*L. collinoides*	26	Y	N	facultative anaerobi	3.6	46.1	DSM 20515	2313	NZ_BBEQ00000000.1	fermented fruits	fermenting apple juice
col	*L. paracollinoides*	25	Y	N	facultative anaerobi	3.5	46.8	DSM 15502	2435	NZ_BBAG00000000.1	fermented cereal	brewery environment
col	*L. similis*	35	Y	N	microaerophilic	3.5	46.9	DSM 23365	2574	NZ_BBAD00000000.1	plants/vegetables/fo	fermented cane molasses at alcohol, plants
com	*L. composti*	30	Y (growth at 10	N	microaerophilic	3.4	43.9	DSM 18527	2822	NZ_BAMK00000000.1	fermented cereal	composting material, of distilled shochu residue
dex	*L. dextrinicus*	30				1.8	38.0	DSM 20335	1667	NZ_AYYK00000000.1	silage	silage
fl	*L. floricola*	30	Y	n/a	facultative anaerobi	1.3	34.5	DSM 23037	1210	NZ_AYZL00000000.1	plants/flowers	flower of Caltha palustris
per	*L. harbinensis*	37	n/a	n/a	microaerophilic	3.1	53.1	DSM 16991	2894	NZ_AUEH00000000.1	fermented vegetable	chinese traditional, fermented vegetable Suan cai
per	*L. perolens*	28	Y	U to 42 °C	facultative anaerobi	3.3	49.1	DSM 12744	3003	NZ_AZEC00000000.1	fuits	orange lemonade
per	*L. shenzhenensis*	37	n/a	Y	facultative anaerobi	3.2	56.4	LY 73	2862	NZ_AVAA00000000.1	dairy	fermented dairy beverage
sak	*L. curvatus*	30	Y (growth at 2-4	N	facultative anaerobi	1.8	42.0	DSM 20019	1699	NZ_AZDL00000000.1	dairy	milk
sak	*L. fuchuensis*	20	Y	N		2.1	41.8	DSM 14340	1791	NZ_BAMI00000000.1	meat	vacuum-packaged beef
sak	*L. graminis*	30	Y	N		1.8	40.2	DSM 20719	1692	NZ_AYZB00000000.1	silage	grass silage
sak	*L. sakei subsp. carno*	37	Y	N	Microaerophilic	2.0	41.0	DSM 15831	1886	NZ_AZFG00000000.1	meat	fermented meat product
sak	*L. sakei subsp. sakei*	30	Y	N	Microaerophilic	1.9	41.0	DSM 20017	1646	NZ_BAUW00000000.1	fermented cereal	Moto starter of sake
sav	*L. aquaticus*	37	Y	Y	Microaerophilic	2.4	37.4	DSM 21051	2117	NZ_AYZD00000000.1	aquatic sample	surface of a eutrophic freshwater pond
sav	*L. hordei*	30	N	N	facultative anaerobi	2.3	34.8	DSM 19519	2127	NZ_AZDX00000000.1	fermented cereal	malted barley
sav	*L. mali*	30	Y	Y	Microaerophilic	2.7	36.0	DSM 20444	2486	NZ_BACP00000000.1	fuits	apple juice from cider press
sav	*L. suicola*	30	Y	N	Microaerophilic	2.5	38.4	DSM 21376	2039	NZ_BALC00000000.1	plants/veretables	sap of Quercus sp.
sav	*L. vaccinum*	30			Microaerophilic	2.7	36.9	DSM 19971	2407	NZ_AZEG00000000.1	fruits	must of Bobal, grape variety
vac	*L. hokkaidonensis*	25	Y (growth at 4 °C	N	microaerophilic	2.3	38.1	DSM 26202	2233	NZ_AP014680.1	silage	timothy grass (*Phleum pratense* L.) silage
vac	*L. oligofermentans*	25	Y (growth at 4 °C	N	anerobic	1.8	35.5	DSM 15707	1726	NZ_AZFE00000000.1	meat	broiler leg
vac	*L. suebicus*	30	Y	Y	microaerophilic-ana	2.7	39.0	DSM 5007	2339	NZ_BACO00000000.1	fruits	apple mash
vac	*L. wasatchensis*	25				1.9	39.8	DSM 29958	1713	NZ_AWTT00000000.1	dairy	secondary flora in aged cheese
vac	*L. vaccinostercus*	30	N	N		2.6	43.5	DSM 20634	2359	NZ_AYYO00000000.1	feces-vertebrate	cow dung

Table S1.1 (cont.). Metadata of type strains with lifestyle assignment in Fig. 1.2

Group	Species	Optimal growth temperature (°C)	Growth at 15 °C	Growth at 45 °C	Atmosphere	Genome size (Mb)	GC content (%)	Type Strain	CDSs	Genome Accession No.	Main Habitat	Source of Isolation
Nomadic												
cas	*L. paracasei subsp. t*	30	Y	N		2.4	46.4	DSM 20258	2059	NZ_AYYI00000000.1	dairy	pasteurized milk
cas	*L. casei*	30	Y	N		2.8	46.5	DSM 20011	2566	NZ_AZCO00000000.1	dairy	cheese
cas	*L. paracasei subsp. p*	30		some		2.9	46.5	DSM 5622	2779	NZ_AZGH00000000.1	fermented vegetable	Chinese pickle
pla	*L. herbarum*	30				2.9	43.5	DSM 20350	2627	NZ_LFEE00000000.1	fermented vegetable	fermented radish
pla	*L. paraplantarum*	30	Y	N	microaerophilic	3.1	44.0	DSM 10667	2751	NZ_CP013130.1	fermented cereal	beer contaminant
pla	*L. fabifermentans*	30	Y (growth at 10	N	microaerophilic	3.3	45.0	DSM 21115	2973	NZ_AYGK00000000.2	fermented plant	cocoa bean heap fermentation
cas	*L. rhamnosus*	37	Y	Y		2.9	46.7	DSM 20021	2619	NZ_AZCQ00000000.1		-
pla	*L. plantarum subsp.*	30	Y	N	microaerophilic	3.2	45.0	DSM 16365	3013	AZFR00000000	fermented vegetable	fermented cassava roots (fufu)
pla	*L. plantarum subsp.*	37	Y	N		3.2	44.5	ATCC 14917	2892	NZ_ACGZ00000000.2	fermented vegetable	Pickled cabbage
pla	*L. pentosus*	30	Y	N		3.4	46.4	DSM 20314	2915	NZ_CM001538.1	silage	Corn silage
Insect-adapted												
del	*L. bombicola*	37			microaerophilic	1.7	34.6	DSM 28793	1570	FOMN00000000.1	bee	bumble bees
del	*L. kullabergensis* "Fi	30	Y	Y		2.1	35.5	DSM 26262	1883	NZ_JXBY00000000.1	bee	the honey stomach of the honeybee Apis mellifera
del	*L. apis* "Firm 5"	37			microaerophilic	1.7	36.6	LMG 26964	1553	NZ_JXLG00000000.1	bee	bees
del	*L. melliventris* "Firm	30	Y	Y		2.1	35.7	DSM 26256	1959	NZ_JXIJ00000000.1	bee	honey stomach of honey bee (Apis mellifera mellifera)
del	*L. kimbladii* "Firm 5"	30	Y	Y	anaerobic	2.1	35.8	DSM 26263	1955	NZ_JXLH00000000.1	bee	honey stomach of honey bee (Apis mellifera mellifera)
del	*L. helsingborgensis* "	35	Y	Y	anaerobic	2.0	36.4	DSM 26265	1770	NZ_JXIR00000000.1	bee	bee
mel	*L. mellis* "Firm 4"	30	Y	Y	anaerobic	1.8	36.2	DSM 26255	1622	NZ_JXBZ00000000.1	bee	honey stomach of honey bee (Apis mellifera mellifera)
mel	*L. mellifer* "Firm 4"	35	Y	Y	anaerobic	1.8	39.3	DSM 26254	1654	NZ_JXLQ00000000.1	bee	the honey stomach of the honeybee Apis mellifera
kun	*L. ozensis*	30			microaerophilic	1.5	31.9	DSM 23829	1402	NZ_AYXQ00000000.1	plant/flower	inula ciliaris var. glandulosa, a chrysanthemum
kun	*L. apinorum*	35	Y	Y	anaerobic	1.4	34.5	DSM 26257	1292	NZ_JXCT00000000.1	bee	bees
kun	*L. kunkeei*	30	Y	N	microaerophilic	1.5	36.4	DSM 12361	1328	NZ_AZCK00000000.1	bee, plant/flower/fe	commercial, grape wine undergoing a sluggish/stuck alcoholic fermentation
fru	*L. sanifranciscensis*	30	Y	N	microaerophilic - an	1.3	34.7	DSM 20451	1234	NC_015978.1	fermented cereal	San Francisco sourdough
fru	*L. hormohiochii*	26	Y	N	microaerophilic	1.4	38.8	DSM 20571	1301	NZ_JQBW00000000.1	fermented meat	Portuguese traditional dry fermented sausage
fru	*L. florum*	30	Y	N	microaerophilic	1.3	41.1	DSM 22689	1273	NZ_AYZI00000000.1	plant/flower	peony (Paeonia suffruticosa)
fru	*L. fructivorans*	26	Y	N	microaerophilic	1.4	38.9	DSM 20203	1283	NZ_AEQY00000000.1	silage	alfalfa silage

Table S1.1 (cont.) Metadata of type strains with lifestyle assignment in Fig. 1.2

Group	Species	Optimal growth temperature (°C)	Growth at 15 °C	Growth at 45 °C	Atmosphere	Genome size (Mb)	GC content (%)	Type Strain	CDSs	Genome Accession No.	Main Habitat	Source of Isolation
Vertebrate-adapted												
del	*L. equigenerosi*	37	N	Y		1.6	42.6	DSM 18793	1186	NZ_BBAS00000000.1	horse	thoroughbred horses
del	*L. hamsteri*	37	N			1.8	35.0	DSM 5661	1581	NZ_BAUY00000000.1	hamster	faeces of hamster
del	*L. amylolyticus*	45	N	Y		1.5	38.2	DSM 11664	1406	NZ_ADNY00000000.1	pig, fermented cerea	acidified beer wort
del	*L. gasseri*	37	N	Y	aerobic	1.8	35.0	ATCC 33323	1567	NZ_BALQ00000000.1	-	-
del	*L. gallinarum*	37	Y	Y		1.9	36.4	DSM 10532	1760	NZ_AZEL00000000.1	chicken	chicken crop
del	*L. johnsonii*	37	Y	Y		1.8	34.5	DSM 10533	1575	NZ_ACGR00000000.1	rodent	human blood
del	*L. taiwanensis*	37	N	Y	microaerophilic	1.9	34.0	DSM 21401	1662	NZ_AYZG00000000.1	silage	silage cattle feed
del	*L. kitasatonis*	37	N	Y	microaerophilic	1.9	37.5	DSM 16761	1749	NZ_AZFU00000000.1	chicken	chicken, intestine
del	*L. amylovorus*	37	N	Y	microaerophilic - ana	2.0	37.8	DSM 20531	1805	NZ_AZCM00000000.1	cattle/pig	cattle / pigs
del	*L. gigeriorum*	37	N	Y	microaerophilic	1.9	36.9	DSM 23908	1745	NZ_CAKC00000000.1	chicken	chicken crop
del	*L. crispatus*	37	N	Y	anaerobic	2.0	36.9	DSM 20584	1840	NC_014106.1	human vagina	eye
del	*L. equicursoris*	37	N	Y	microaerophilic	2.1	47.7	DSM 19284	1825	NZ_AZDU00000000.1	horse	healthy thoroughbred racehorse
del	*L. ultunensis*	37	N		anaerobic	2.2	36.0	DSM 16047	1947	NZ_ACGU00000000.1	human	gastric biopsies, human stomach mucosa
del	*L. intestinalis*	37	N	Y	microaerophilic	2.0	35.3	DSM 6629	1730	NZ_AZGN00000000.1	rat	intestine of rat
del	*L. jensenii*	37	N	Y	microaerophilic - ana	1.6	34.4	DSM 20557	1363	NZ_ACGQ00000000.2	human	human vagina, discharge
del	*L. kalixensis*	37	N	Y	anaerobic	2.1	36.1	DSM 16043	1859	NZ_AZFM00000000.1	human	gastric biopsies, human stomach mucosa
del	*L. posteurii*	37	N	Y	microaerophilic	1.9	38.6	DSM 23907	1718	NZ_CAKD00000000.1	chicken	chicken crop
del	*L. hominis*	37	N	Y		1.9	35.1	DSM 23910	1773	NZ_CAKE00000000.1	human	human intestine
del	*L. psittaci*	37	Y	Y	anaerobic	1.5	35.7	DSM 15354	1308	NZ_AUEI00000000.1	parrot	lung of parrot
del	*L. iners*	37	N		anaerobic	1.3	32.5	DSM 13335	1165	NZ_ACLN00000000.1	human vagina	human urine
del	*L. helveticus*	37	N	Y	microaerophilic - ana	2.2	36.8	DSM 20075	1913	NZ_ACLM00000000.1	chicken	chickens
reu	*L. vaginalis*	37	N	Y		1.8	40.6	DSM 5837	1634	NZ_ACGV00000000.1	human	vagina, swab from patient with trichomoniasis
reu	*L. oris*	37	N	Y		2.0	50.0	DSM 4864	1826	AZGE00000000	human	human saliva
reu	*L. panis*	37	N	Y	anaerobic	2.0	48.1	DSM 6035	1753	NZ_AZGM00000000.1	fermented cereal	sourdough
reu	*L. pontis*	30	Y	Y	microaerophilic	1.7	54.4	DSM 8475	1502	NZ_AZGO00000000.1	pig, chicken	pigs and chicken
reu	*L. coleohominis*	37	N	Y		1.7	40.8	DSM 14060	1570	AZEW01000000	human	human vagina
reu	*L. reuteri*	37	N	Y	anaerobic	1.9	38.6	DSM 20016	1846	NZ_AZDD00000000.1	human, rodent, chic	intestine of adult
reu	*L. gastricus*	37	N	N (max 42°C	microaerophilic-anae	1.8	41.6	DSM 16045	1727	NZ_AZFN00000000.1	human	gastric biopsies, human stomach mucosa
reu	*L. ingluviei*	42	N	Y	microaerophilic-ana	2.0	50.9	DSM 15946	1993	NZ_AZFK00000000	pigeon	pigeon, crop
reu	*L. frumenti*	40	N	Y	microaerophilic	1.7	42.5	DSM 13145	1611	NZ_AZER00000000.1	fermented cereal	rye-bran sourdough
reu	*L. antri*	37	N	Y	microaerophilic-anae	2.2	51.1	DSM 16041	2031	NZ_ACLL00000000.1	human	gastric biopsies, human stomach mucosa
reu	*L. senioris*	37	Y	N	microaerophilic	1.6	39.1	DSM 24302	1528	NZ_AYZR00000000.1	human	feces of a healthy 100-year-old Japanese female
reu	*L. gorillae*	37			microaerophilic - ana	1.6	48.1	DSM 28356	1582	BCAH01000001 to BCAH0100	gorilla	gorilla
reu	*L. mucosae*	37	N	Y		2.3	46.4	DSM 13345	2067	NZ_AZEQ00000000.1	pig	pig small. intestine
sav	*L. equi*	37	Y	N	microaerophilic	2.2	39.0	DSM 15833	1878	NZ_BAMI00000000.1	horse	faeces of horses
sav	*L. oeddipiscis*	30	N	N	microaerophilic	2.3	39.1	DSM 15836	2002	NZ_AZFI00000000.1	fermented fish	fermented fish
sav	*L. hayakitensis*	37	N	Y	anaerobic	1.7	34.0	DSM 18933	1458	NZ_BAML00000000.1	horse	faeces of thoroughbred (horse)
sav	*L. ruminis*	37	N	Y	anaerobic	2.1	43.5	DSM 20403	1836	NC_015975.1	cattle	bovine rumen
sav	*L. murinus*	37	N	Y	n/a	2.2	40.0	DSM 20452	1938	NZ_AYYN00000000.1	rat	intestine of rat
sav	*L. apodemi*	37	N	Y	microaerophilic-anae	2.1	38.6	DSM 16634	1913	NZ_AZFT00000000.1	mouse	feces, wild Japanese wood mouse
sav	*L. saerimneri*	37	W (weak)	Y	anaerobic	1.7	42.5	DSM 16049	1621	NZ_AUHO00000000.1	pig	pig faeces
sav	*L. agilis*	37	N	Y	microaerophilic - ana	2.1	41.7	DSM 20509	1960	NZ_CVQY00000000.1	sewage	municipal. sewage
sav	*L. salivarius*	37	N	Y		2.0	32.5	DSM 20555	1858	NZ_ACGT00000000.1	human saliva	saliva
sav	*L. animalis*	37	N	Y		1.9	41.1	DSM 20602	1685	NZ_AEOF00000000.1	baboon	dental. plaque of baboon
sav	*L. ceti*	37	Y (slow)	N	microaerophilic	1.4	33.7	DSM 22408	1236	NZ_AUHP00000000.1	whale	beaked whales (*Ziphius cavirostris*)
sav	*L. aviarius subsp. arc*	37	N		anaerobic	1.5	40.1	DSM 20653	1370	NZ_AYYZ00000000.1	chicken	intestine of chicken
sav	*L. aviarius subsp. avi*	37	N		anaerobic	1.7	40.1	DSM 20655	1529	NZ_AYZA00000000.1	chicken	faeces of chicken

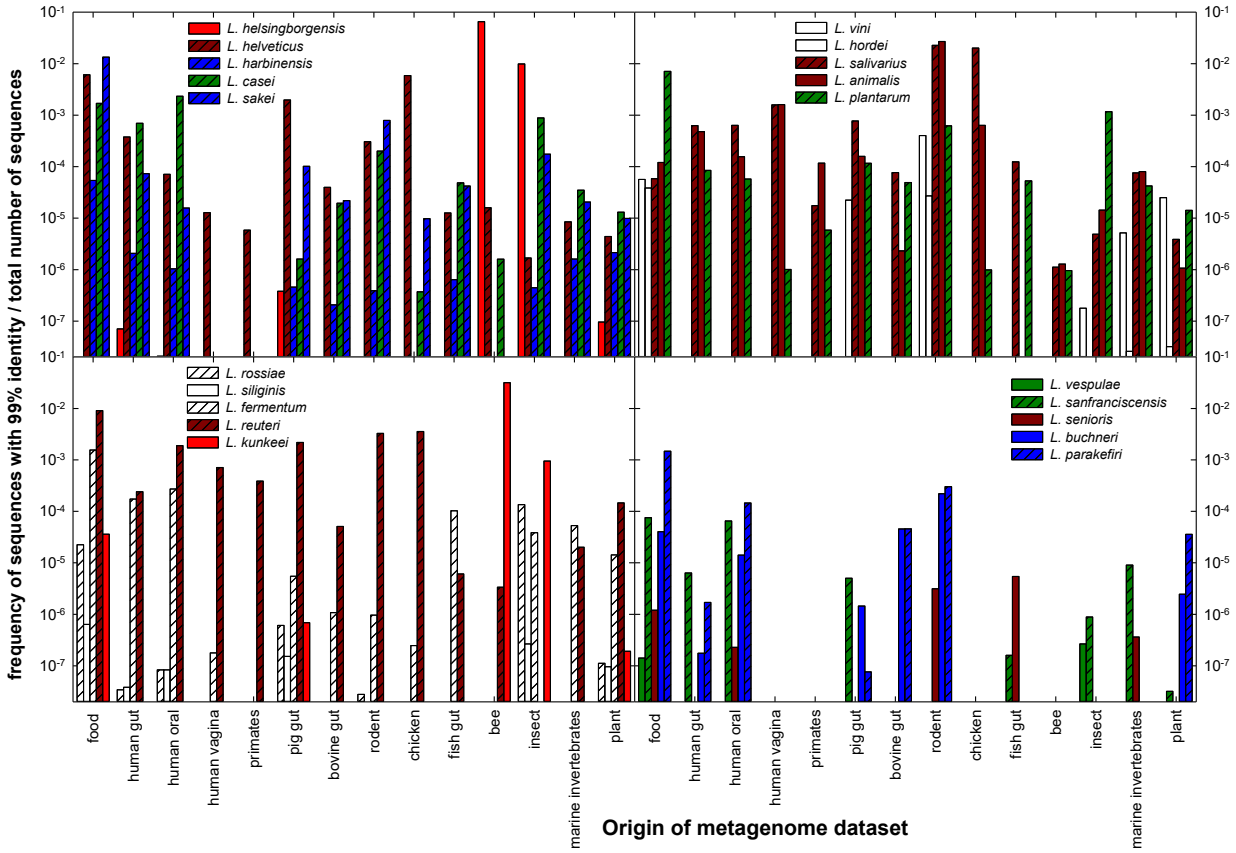


Figure S1. Frequency of selected Lactobacillus species in food, animals, and the environments. Full length 16S rRNA sequences of the species shown were used as query sequence to search the metagenome database at www.imngs.org. In January 2017, the database contained 88580 metagenome datasets with a total of 2,565,966,305 sequences; the selected categories shown represent 1,014,194,428 sequences.

Colours represent lactobacilli adapted to vertebrate hosts (dark red), insects (light red), nomadic lactobacilli (green), or environmental lactobacilli (blue). Lactobacilli with unknown habitat are without colour; hatched bars represent food-fermenting lactobacilli.

The category “food’ includes the IMNGS classifications “food” and “fermentations”; the category “rodent” includes “mouse gut and skin”, “rat gut” and “rodent”; the category “insect” includes “insect gut”, “mosquito” and “termites”; the category “marine invertebrates” includes “coral metagenome”, “echinoderm”, “jellyfish”, “mollusc”, “sea squirt”, “shrimp” and “sponge”; the category “plant” includes “compost”, “endophyte”, “leaf”, “phyllosphere”, “plant”, “root and associated fungi”, and “shoot”. Other categories were omitted because the frequency of sequences matching *Lactobacillus* sequences was below 10-5, or because the sequences were obtained from unclassified or artificial habitats.

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2. Chapter two: Experimental evaluation of host adaptation of *Lactobacillus reuteri* to different vertebrate species

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2.1 Introduction

Vertebrates have gained access to a number of metabolic functions absent in their genomes by virtue of symbiosis with microbes (Moran 2006). The crop and the cecum are evident anatomical adaptations to exploit the enzymatic activity of trillions of symbiotic microbes to access nutrients from otherwise indigestible diets (Stevens and Huma 1998). In addition to nutrient provision, gut microbes exclude pathogens and aid in development of the host's immune system (Dethlefsen, McFall-Ngai and Relman 2007). It is clear vertebrates benefit from symbiotic associations with microbes. However, core concepts on how symbioses are formed and maintained over evolutionary time scales are not well understood.

The taxonomic profile of the vertebrate microbiota is largely host-specific (Dethlefsen, McFall-Ngai and Relman 2007; Ley *et al.* 2008a) and in some cases, congruent with the evolution of the host species (Ochman *et al.* 2010). This apparent relatedness between microbial community composition and host phylogeny has been interpreted as evidence for co-evolution (Ley *et al.* 2008b; Fraune and Bosch 2010; Rosenberg and Zilber-Rosenberg 2016). Much of this information has been derived from analyzing 16S rRNA gene sequences which have evolved too slowly to provide information on evolutionary relationships over relevant time scales, especially given the more recent diversification of contemporary vertebrate species compared to their bacterial symbionts (Walter, Britton and Roos 2011). Examples of co-diversification of specific bacterial lineages with mammalian hosts have been discovered by analyzing fast-evolving gene phylogenies (Falush *et al.* 2003; Moeller *et al.* 2016) suggesting co-speciation of some symbiotic microbes alongside their vertebrate hosts. However, even in the few established cases, the mechanisms by which these microbes evolved and the outcomes that arose from the evolutionary process remain undefined.

Symbiotic gut microbes that remain stably associated with particular vertebrate species are predicted to evolve host-specific adaptations and as a result, display enhanced ecological performance in their cognate host (Garcia and Gerardo 2014). Determining the rate of colonization success of individual symbionts in naïve (aposymbiotic) hosts in combination with phylogenetic analyses thus provides a platform to infer the evolutionary mechanisms by which host-microbe symbioses evolve. Such experimental approach has been successfully applied to study the evolutionary consequences of bacterial symbioses in insects (Dale and Moran 2006; Kwong *et al.* 2014) and in invertebrates (Wollenberg and Ruby 2012; Bongrand *et al.* 2016), but rarely in vertebrates.

The bacterial species *Lactobacillus reuteri* inhabits the gastrointestinal tracts of a variety of vertebrates and has diversified into distinct phylogenetic lineages that are coherent with host origin (Oh *et al.* 2010). A series of phylogenetic, phylogenomic and experimental studies in mice have established this species as a paradigm for host adaptation of a non-pathogenic symbiont of the vertebrate gut microbiota (Walter, Britton and Roos 2011). Rodent isolated strains display elevated fitness in mice (Oh *et al.* 2010; Frese *et al.* 2011) and biofilm formation in the forestomach is restricted to strains from rodent lineages (Frese *et al.* 2013). Together these results show that specialized adaptations to the gut environment of the host underlie the evolution of *L. reuteri* with rodents. In contrast, the mechanisms by which *L. reuteri* has evolved with other vertebrate host species have not been determined. In this respect, it is important to point out that the presence of host-specific lineages in itself does not provide evidence for natural selection as clusters can arise by neutral processes such as genetic drift (Doolittle and Zhaxybayeva 2009; Oh *et al.* 2010). Furthermore, clustering of both human and poultry isolates in the same phylogenetic lineage (lineage-VI) has raised questions regarding the evolutionary history of this lineage. Therefore, the goals of this study were to test for host adaptation of *L. reuteri* to non-rodent hosts and to resolve outstanding questions regarding the evolution of the phylogenetic

lineage-VI. We developed and validated an experimental method to systematically compare the ecological performance of strains isolated from different hosts and assigned to distinct phylogenetic lineages, in the gastrointestinal tracts of chicken, pigs and human volunteers. We then complemented these studies with comparative genomic analyses to gain insight into genome evolution of poultry and human strains of the lineage-VI.

2.2 Materials and Methods

2.2.1 Strains, media and growth conditions

Bacterial strains used in this study are listed in Table 2.1 and were grown at 37 °C in deMan Rogosa Sharpe medium supplemented with 10 g/l maltose and 5 g/l of fructose (mMRS). To ensure selective cultivation from feces, a *L. reuteri* isolation medium (LRIM) was devised based on the recipe of the Rogosa medium (Rogosa, Mitchell and Wiseman 1951). The LRIM contained per liter, 15 g of raffinose, 15 g of sodium acetate, 15 g of agar, 10 g of tryptone, 6 g of KH_2PO_4, 5 g of yeast extract, 2 g of ammonium acetate, 1.32 ml of glacial acetic acid, 1 g of tween 80, 0.57 g of $MgSO_4$, of 0.12 g of $MnSO_4$, 0.003 g of $FeSO_4$. Since only a limited number of lactobacilli species grow on raffinose (Pot *et al.* 2014), this media allowed for a sufficiently selective culture of *L. reuteri* in the presence of a background fecal microbiota. Incubations in LRIM were performed under anaerobic conditions (<0.1 % O_2 ; ≥15% CO_2) for 48 h at 45 °C.

2.2.2 Preparation of strain mixtures to prepare inocula

Eighteen *L. reuteri* strains originating from different hosts and assigned to separate phylogenetic lineages were selected and divided into three mixtures (see Table 2.1). To facilitate differentiation, strains within the same inoculum were selected to carry distinct *leuS* alleles. Inocula for host experiments were prepared by growing individual stains overnight in mMRS, followed by subculture (with 1% inoculum each) twice in previously boiled (100 °C for 30 min) food-grade DE-PHAGE® media (Cargill) supplemented with 20 g/l of malt. Cell numbers of each

individual strain after growth for 16 h in DE-PHAGE® media were determined in at least six replicate experiments. This information was used to prepare standardized inocula with equivalent proportions of each strain and adjusted to contain approximately 3 x 10^5 cells of total *L. reuteri* per gram of host body weight (BW). In order to determine if these conditions were met, cell numbers of each individual stain were determined by quantitative culture on mMRS, prior to mixing the inoculums

Table 2.1- *L. reuteri* strains used in the host adaptation assay

	Strain	Host of isolation	Lineage[a]	*leuS* allelic profile[a]
Inoculum A				
	Cf46g	Human	II	4
	mlc3	Mouse	III	31
	CR	Rat	I	14
	CSF8	Chicken	VI	15
	M27U15	Human	VI	11
	JW2015	Pig	IV	3
Inoculum B				
	MM4-1a (ATCCPTA 6475)	Human	II	4
	r2lc	Rat	III	35
	lpuph1	Mouse	I	9
	1366	Chicken	VI	6
	CF48-3A	Human	VI	11
	lpa1	Pig	IV	3
Inoculum C				
	sr11	Human	II	4
	n2d	Rat	III	34
	6799jm1	Mouse	I	9
	JCM1081	Chicken	VI	24
	MM34-4A	Human	VI	11
	ATCC 53608	Pig	IV	3

[a] Lineage and *leuS* type as determined in (Oh *et al.* 2010)

2.2.3 Mouse experiment

Germ-free C3H/HeN mice (~30 g BW) were maintained at the University of Nebraska Gnotobiotic Mouse Facility. Groups of mice (n=5) were assigned to receive one of the three inocula and subsequently moved from sterile isolators into individual ventilated biocontainment cages. To prepare the inocula, *L. reuteri* strains grown for 16 h on DE-PHAGE® media were harvested by centrifugation (3000 $\times$ *g* for 10 min) and washed twice with sterile PBS (pH 7.0). Each mouse was gavaged with 100 µl of PBS suspension containing a total of 3 x 10^5 cells per gram of BW. *L. reuteri* were enumerated on LRIM from fresh fecal pellets collected immediately prior and daily for 5 days after gavage. To ensure gnotobiotic conditions were maintained during the experiments, three mice were gavaged with sterile PBS and housed in separate biocontainment cages located in the same ventilator rack as the experimental mice. Fecal pellets from control mice were plated daily on BHI and mMRS media and checked for aerobic and anaerobic growth. All procedures were conducted with approval from the Institutional Animal Care and Use Committee of the University of Nebraska (Project ID 731).

2.2.4 Chicken experiment

Specific pathogen free Leghorns were hatched in the Poultry Research Facility at the University of Alberta and transported the same day to the Animal Research Facility of the same institution. Birds were randomly assigned to an inoculum (n=5 per inoculum) or PBS control (4 birds). Chickens were housed in pairs or groups of three and maintained in biocontainment cages. In order to obtain *Lactobacillus*-free (LF) chickens penicillin was added to the drinking water at a concentration of 0.6 g/l. Four days after days of penicillin treatment commenced, absence of lactobacilli was confirmed by plating cloacal swabs on Rogosa (Difco) and LRIM. Antibiotic administration was removed 18 h prior to LF-chickens (~ 30g BW) being gavaged with 300 µl of PBS containing standardized *L. reuteri* inocula with a total of 3 $\times$ 10^5 cells per gram of BW. Cloacal

samples were obtained from each animal immediately prior (day 0) and for five days after gavage. Upon collection, cloacal samples were transferred into 1.5 ml tubes containing sterile PBS with 10% v/v glycerol and immediately processed by dilution plating on LRIM. All procedures were carried out in accordance with protocol AUP00000003 approved by the University of Alberta's Animal Care and Use Committee.

2.2.5 Pig experiment

Nine germ-free piglets were delivered from a full-term pregnant sow by sterile hysterectomy following methods described by Miniats and Jol (Miniats and Jol 1978) and aseptically transferred into one of three sterile polyvinyl flexible isolators. Animals in the same isolator (n=3) were kept in separate stainless steel compartments with false floors to collect excreta. Isolators were maintained under positive pressure at an ambient temperature of 35°C and ventilated with sterilized filtered air pre-warmed to the same temperature. Piglets were fed commercially sterile infant formula throughout the experiment. At 10 days of age, piglets (~3 Kg BW) in the same isolator were administered either inocula A, B or C containing *L. reuteri* at 3×10^5 cells per gram of BW and suspended in the feeding infant formula. Cell counts of *L. reuteri* were enumerated by quantitative LRIM culture of fecal samples obtained directly from the pig's rectum using sterile plastic loops and collected before (day 0) and at days 1 to 5 post-administration. Fecal samples were also plated on BHI to detect any contamination. Aerobic growth on BHI was detected in the feces of one of the treatment groups on day 3 to 5 post-inoculation. However, *L. reuteri* counts were not different when compared with pigs in the other groups. All procedures were conducted in with approval of the Institutional Animal Care and Use Committee of the University of Nebraska (Project ID 939).

2.2.6 Human subjects

Fecal samples from twenty human subjects were screened for growth on LRIM. Of those, fifteen (eight females, seven males) produced less than 10^4 CFU per gram of feces and were considered eligible for the study. Subjects were then randomly assigned to receive one of the three inocula (n=5 per inoculum). All subjects were between the ages of 18 and 55, abstained from using probiotic and prebiotic products, had not consumed oral antibiotics within 3 months before the study and considered themselves healthy.

Inocula were prepared in the food laboratory of the Department of Agricultural, Food, and Nutritional Science at the University of Alberta. Briefly, *L. reuteri* strains were grown for 16 h on DE-PHAGE® media and mixed to contain a total of 3×10^5 cells per gram of BW (considering an average BW of 75 kg). Cells were harvested by centrifugation at 3000 $\times g$ for 5 min and suspended in bottled spring water immediately prior to consumption. Subjects were instructed to drink contents of the solution in a single setting. Fecal samples were collected daily from day 0 (before consumption) and during days 1 to 5 post-consumption. Samples were processed within 2 h of deposition and *L. reuteri* was cultured by quantitative dilution plating on LRIM. Human studies were completed at the University of Alberta in accordance to the protocol Pro00051493 approved by The Health Research Ethics Board - Biomedical panel.

2.2.7 Strain typing and identification.

Sixteen colonies were randomly selected from LRIM plates of each fecal sample (or cloacal swab) cultured from day 1 to day 5 post-inoculation and typed by colony PCR. The *leuS* gene was directly amplified from each colony with the primers *leuS*-F TACGACGCGGGCAGATAC and *leuS*-R ATAGAGATCAACTGGTGACC. PCR conditions described previously (Oh *et al.* 2010) were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1 min, with a final extension of 7 min at 72°C. PCR products were purified using the QIAquick

PCR Purification Kit (Qiagen) and sequenced using Sanger technology. Sequences were assigned to a strain by BLASTn search against a local nucleotide database implemented in Bioedit (Hall 1999).

2.2.8 Genome sequencing and annotation

Genomic DNA from the strains *L. reuteri* 1366, CSF8, JCM1081 (poultry-VI), and MM34-4A, M27U15 and CF48-3A1 (human-VI) was obtained using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) with some modifications as described by Oh et. al (2010) (Oh *et al.* 2010). Genomes were sequenced to draft status at The Applied Genomics Centre (TAGC, University of Alberta) using Illumina MiSeq paired-end technology. Reads were assembled into scaffolds with SPADES (Bankevich *et al.* 2012) available in PATRIC (Wattam *et al.* 2014), annotated using the Joint Genome Institute (http://jgi.doe.gov) pipeline and deposited in the Integrated Microbial Genomes system (IMG) (Markowitz *et al.* 2008).

2.2.9 Accession numbers

The genome sequences of *L. reuteri* sequenced in this study were deposited in the Integrated Microbial Genomes system (IMG) (Markowitz *et al.* 2008). Genome IDs are provided in Table 2. Genome sequences are also available in the GenBank under the BioProject ID: PRJNA380292.

2.2.10 Comparative genomic and phylogenetic analyses

Comparative analysis of genome sequences was performed in EDGAR (Blom *et al.* 2016) based on an all-against-all comparison of the predicted proteomes (GenBank) downloaded from the JGI. Unique and lineage-specific genes were determined using the “singleton” and “genesets” functions in EDGAR (Blom *et al.* 2016) and confirmed with the IMG phylogenetic profiler (Markowitz *et al.* 2008). Single nucleotide polymorphisms were detected with Mauve (Darling, Mau and Perna 2010) and the average nucleotide identity (ANI) in EDGAR (Blom *et al.* 2016).

The core genome was calculated as the set of orthologous genes present in all strains by bidirectional best BLAST hits. Phylogeny was constructed by aligning 900 core orthologous genes present in all the genomes sequenced in this study and others available in the public databases (Table 1). Concatenated sequences were used to calculate a distance matrix, which provided the input for the neighbor-joining method in the PHYLIP package as implemented in EDGAR (Blom *et al.* 2016). Tree was drawn and annotated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/)

2.2.11 Ancestral state analysis

To infer the order of emergence of host lineages, the sequences of the seven gene of 116 strains used in previous multi-locus sequence analysis (Oh *et al.* 2010) were analyzed using Mesquite 3.2 (Maddison and Maddisson 2017) as previously described (Sachs, Skophammer and Regus 2011). *Lactobacillus fermentum* was used as an outlier. The host of each strain was assigned as rodent, swine, poultry or human based on the origin of isolation (Oh *et al.* 2010). Ancestral states were inferred using parsimony. Host switch events were identified when two or more equally parsimonious ancestral state reconstructions were found, or when the host state of the immediate ancestral node was different from the offspring. The putative dates of host switch events were estimated using Bayesian phylogenetic analyses in BEAST v.2.4.3 (Bouckaert *et al.* 2014) using the HKY85 substitution model, an estimated clock rate of 10^{-8} and the Calibrated Yule model.

2.2.12 Statistical analysis

Statistical significance was determined by one-way analysis of variances (ANOVA) with Tukey's post hoc test ($\alpha = 0.05$) implemented in the statistical package GraphPad Prism version 6.0 (GraphPad Software, La Joya, CA, USA).

2.3 Results

2.3.1 Evolutionary relationships of *L. reuteri* strains using whole genome phylogenetic analysis

To achieve a higher resolution in the analysis of the evolutionary relationships of strains belonging to lineage-VI, we sequenced the genomes of six strains from this cluster (3 originating from chickens and 3 from humans) and included these genomes in a whole-genome phylogenetic

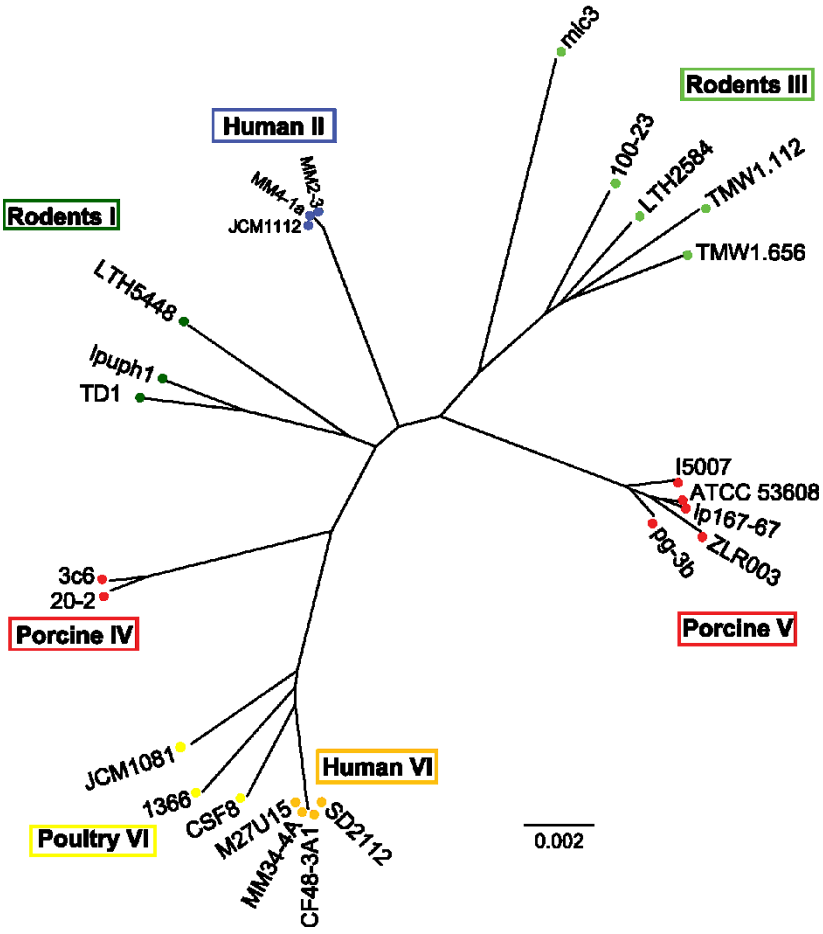

Figure 2.1 Neighbor-joining tree of *Lactobacillus reuteri*

Phylogenetic tree of *Lactobacillus reuteri* based on core genome alignment (900 genes) of 25 strains. Tips of the branches are color coded by lineage and cohesive clades are labeled.

analysis with an additional nineteen *L. reuteri* genomes (Table 2). As shown in Fig. 2.1, the tree showed clear separation of *L. reuteri* into six host-defined phylogenetic lineages in complete agreement with the previous multi-locus sequencing analysis (Oh *et al.* 2010). Whole-genome phylogenetic analysis further confirmed human and poultry isolates clustered in lineage VI, which we refer to as human-VI and poultry-VI strains, respectively. This analysis also revealed important differences in genome diversity among lineage-VI strains, with the genomes of human-VI clustered tightly while poultry-VI strains showed high diversity (Fig. 2.1).

2.3.2 Introduction of *L. reuteri* to the digestive tract of different vertebrate hosts.

We developed an experimental approach to study host adaptation of *L. reuteri* strains in different hosts (Fig. 2.2). We designed three inocula each containing six *L. reuteri* strains representing lineage I, II, III, V and VI, with isolates originating from rodents (mice or rats), pigs, chicken, and humans, including one chicken-VI strain and one human-VI strain in each inoculum (Table 2.1). We administrated the same three inocula at a standardized dose to germ-free mice and germ-free pigs, LF-chickens and to human volunteers with low background of lactobacilli,) and determined cell numbers before (day 0) and daily for 5 days after administration by quantitative culture on LRIM plates. As shown in Fig. 2.3, *L. reuteri* became detectable between 6.0 – 8.0 $\log_{10}$ CFU/g of feces (or per swab) in all hosts within one day of oral administration. In ex-germ-free mice, ex-LF-chickens and ex-germ-free pigs, *L. reuteri* established populations reaching numbers comparable to those of *Lactobacillus* in conventional animals (Tannock 1992; Leser *et al.* 2002; Abbas Hilmi *et al.* 2007). In contrast, *L. reuteri* became detectable in human fecal samples at around 7.0 $\log_{10}$ CFU/g at day 1 and 2 and subsequently followed by a continuous decline, decreasing to numbers that were just above those of the baseline by day 5. These results were expected as similar temporal patterns were previously observed for the persistence of *L. reuteri* in humans (Frese, Hutkins and Walter 2012; Rattanaprasert *et al.* 2014).

Table 2.2 *L. reuteri* genomes used for phylogeny reconstruction and comparative genomics

Strain (alterative name)	Origin	Lineage	Source
mlc3	mouse	III	JGI: 2506381016
100-23	rat	III	JGI: 2500069000
LTH2584	sourdough	I	JGI: 2534682349
TMW1.112	sourdough	III	JGI: 2534682347
TMW1.656	sourdough	III	JGI: 2534682350
I5007	pig	IV	GenBank: CP006011-CP006017
ATCC 53608	pig	IV	EMBL: LN906634
lp167-67	pig	IV	JGI: 2599185361
ZLR003	pig	N.D	JGI: 2687453552
pg-3b	pig	IV	JGI: 2599185334
3c6	pig	V	JGI: 2599185333
20-2	pig	V	JGI: 2599185332
TD1	rat	I	GenBank: CP006603
lpuph1	mouse	I	JGI: 2506381017
LTH5448	sourdough	I	JGI: 2571042361
JCM1112(DSM20016^T/F275)	human	II	NCBI: NC_01060
MM4-1a (ATCC PTA-6475)	human	II	JGI: 2502171170
MM2-3 (ATCC PTA-4659)	human	II	JGI: 2502171171
CSF8	chicken	VI	JGI: 2684623009
1366	chicken	VI	JGI: 2684623010
JCM1081	chicken	VI	JGI: 2684623011
CF48-3A	human	VI	JGI: 2502171173
M27U15	human	VI	JGI: 2687453659
MM34-4A	human	VI	JGI: 2660238834
SD2112 (ATCC 55730)	human	VI	NCBI: NC_015697.1

[a] as determined in (Oh *et al.* 2010). N.D not determined

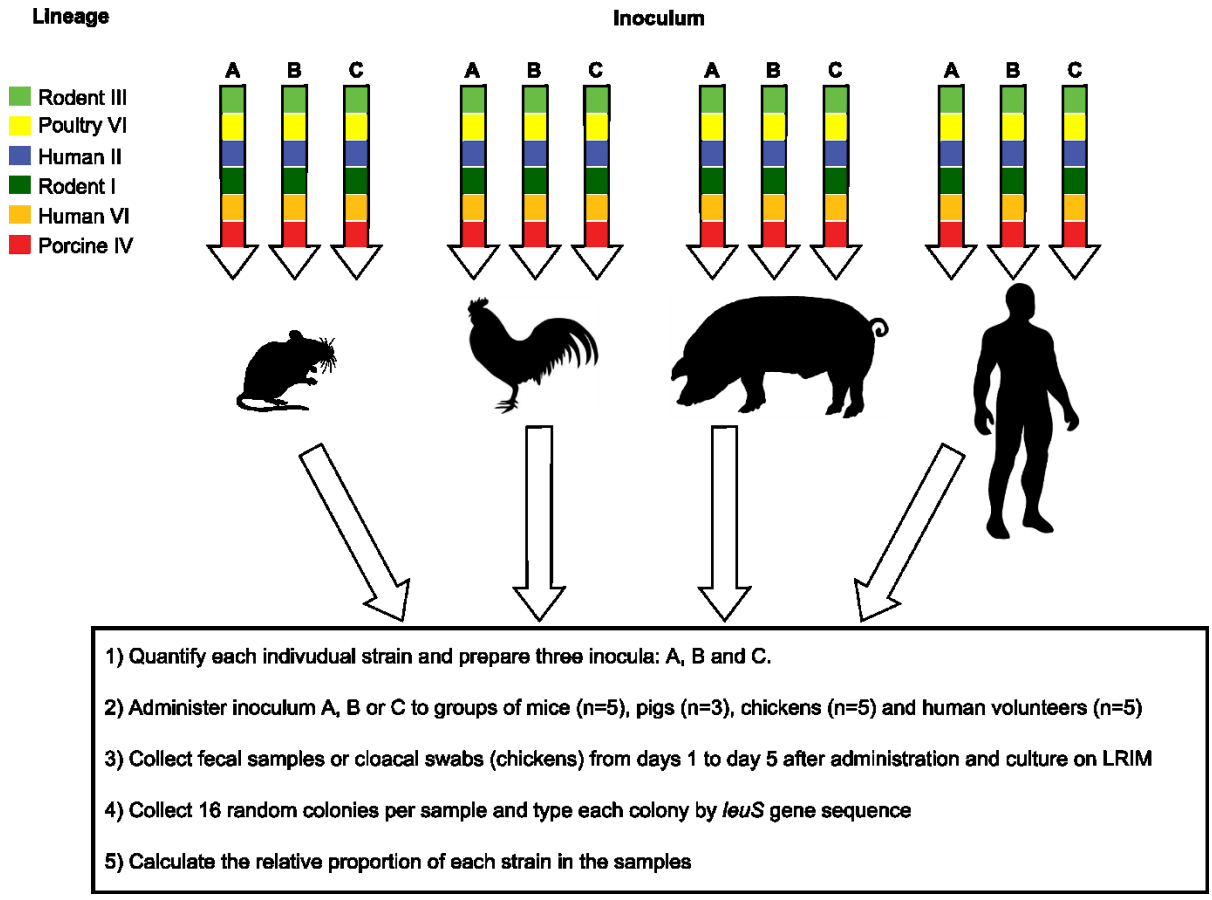

Figure 2.2- Graphic representation of the experimental design.

Eighteen strains of different host origin and phylogenetic lineage were grouped into 3 different inocula containing 6 strains each. To facilitate differentiation, strains within the same inoculum were selected to carry distinct *leuS* alleles. Standardized inocula were prepared to contain equivalent cell numbers of each strain and administered to germ-free mice (n=5 per inocula), *Lactobacillus*-free chickens (n=5 per inocula), germ-free pigs (n=3 per inocula) and humans with a low background of lactobacilli (n=5 per inocula). Bacteria were cultured from the inoculums and from fecal samples collected between day 1 to 5 after administration, and strain composition was determined by randomly typing colonies

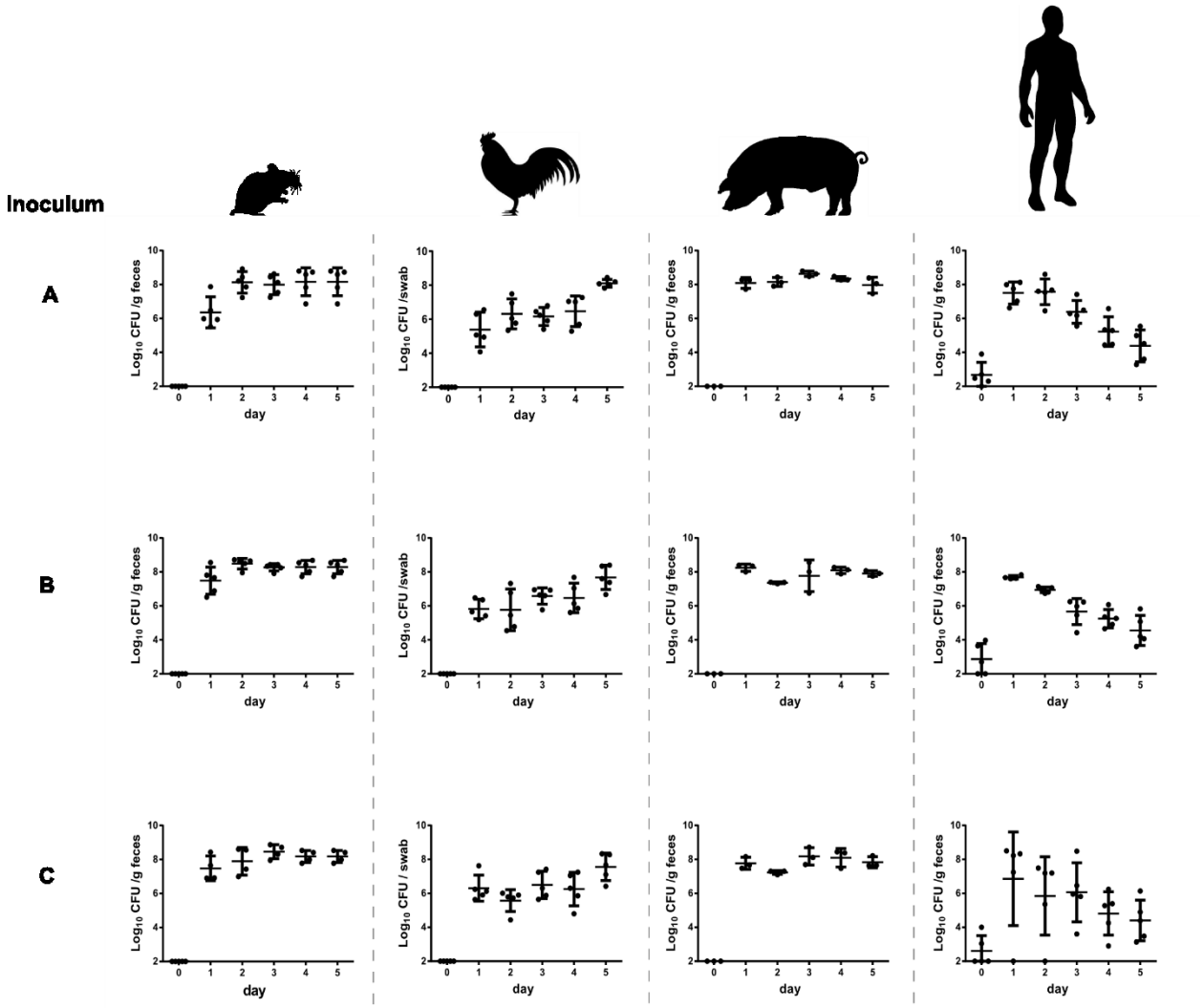

Figure 2.3 Cell numbers of *L. reuteri* in fecal samples (mice, pigs, humans) or cloacal swabs (chickens) determined by quantitative culture

Data are presented as log10 CFU. Each data point represents a sample from individual animals or human volunteers and horizontal bars represent mean ± SD values.

2.3.3 Rodent isolates become enriched in the murine host

Host adaptation of *L. reuteri* strains to mice had been previously established (Oh *et al.* 2010; Frese *et al.* 2011, 2013). Therefore, *L. reuteri* strains from rodents were expected to become enriched in this model. As shown in Fig. 2.4, by day 2 (inocula A and B) or day 3 (inoculum C) the relative abundance of rodent strains was significantly higher ($p< 0.05$) respective to non-rodent lineages. Notably, in those animals receiving inoculum A, nearly all of the colonies typed from days 2 to 5 were identified as the rat isolate CR from the rodent-I lineage. The relative amounts of colonies typed per individual mouse by day 5 are shown in the adjacent bar graphs. As expected, the strain CR was significantly enriched ($p< 0.05$) in mice that received inoculum A. Furthermore, 66% of the colonies recovered on day 5 from mice administered inoculum B, belonged to rodent lineages and compared to human-II, poultry-VI and porcine-V strains, the rodent-I strain lpuph1 was significantly enriched ($p<0.05$). Following the same trend, the rat isolate N2D of inoculum C represented 67% of the colonies typed on this day, being significantly higher ($p<0.05$) than strains from non-rodent lineages.

Together, these results demonstrated that administration of a mixture of strains and subsequent molecular typing (by sequencing the *leuS* gene) of random colonies from fecal cultures allowed us to determine which *L. reuteri* strains became enriched under competitive conditions. Thus, this experimental approach can be used to make accurate inferences about the ecological performance of different strains *in vivo.*

2.3.4 The chicken host

Molecular typing of *L. reuteri* colonies grown from cloacal swabs of chickens that received inoculum A indicated that from days 3 to 5, the relative abundance of the poultry-VI strain CSF8 was significantly higher ($p<0.05$) than strains belonging to all other lineages (Fig. 2.4). Similarly, in animals that received inoculum B, the poultry-VI strain 1366 represented between 50 and 70%

of the colonies typed from days 2 to 5. At day 3 the relative abundance of 1366 was significantly higher (p<0.05) than all other strains except the human-VI strain CF483A1. The poultry-VI strain JCM1081 did not become significantly enriched; however, it represented between 33 and 55% of the colonies recovered from chickens administered inoculum C. By day 5, as shown in the adjacent bar graphs, the strain CSF8 represented a significantly higher (p<0.05) percent of the colonies typed per animal. Similarly, 1366 was found in significantly higher (p<0.05) compared to the human-II and rodent I and III strains. In animals that received inoculum C, the relative abundance of JCM1081 at day 5 (53%) was significantly higher (p<0.05) than most other lineages, except the human-II lineage.

Overall this analysis revealed that chicken-VI strains were enriched during colonization of the chicken gut. Interestingly, we also found that human–VI strains were good colonizers of this host, especially during the early colonization phase. For example, in birds receiving inoculum A, the relative abundance of poultry-VI and human-VI was not significantly different and together these strains accounted for 80% (42.5 % CSF8 and 37.5% M27U15) of the colonies typed on day 1 and 90% (55 % and 36%) on day 2. No significant difference between the relative abundance of poultry-VI strain 1366 and human-VI strain CF484A1 of inoculum B was found at days 1, 3 and 5. Additionally, at day 1, the relative abundance of the human-VI isolate MM344A from inoculum C was slightly higher (43%) than the poultry isolate JCM1081 (33 %).

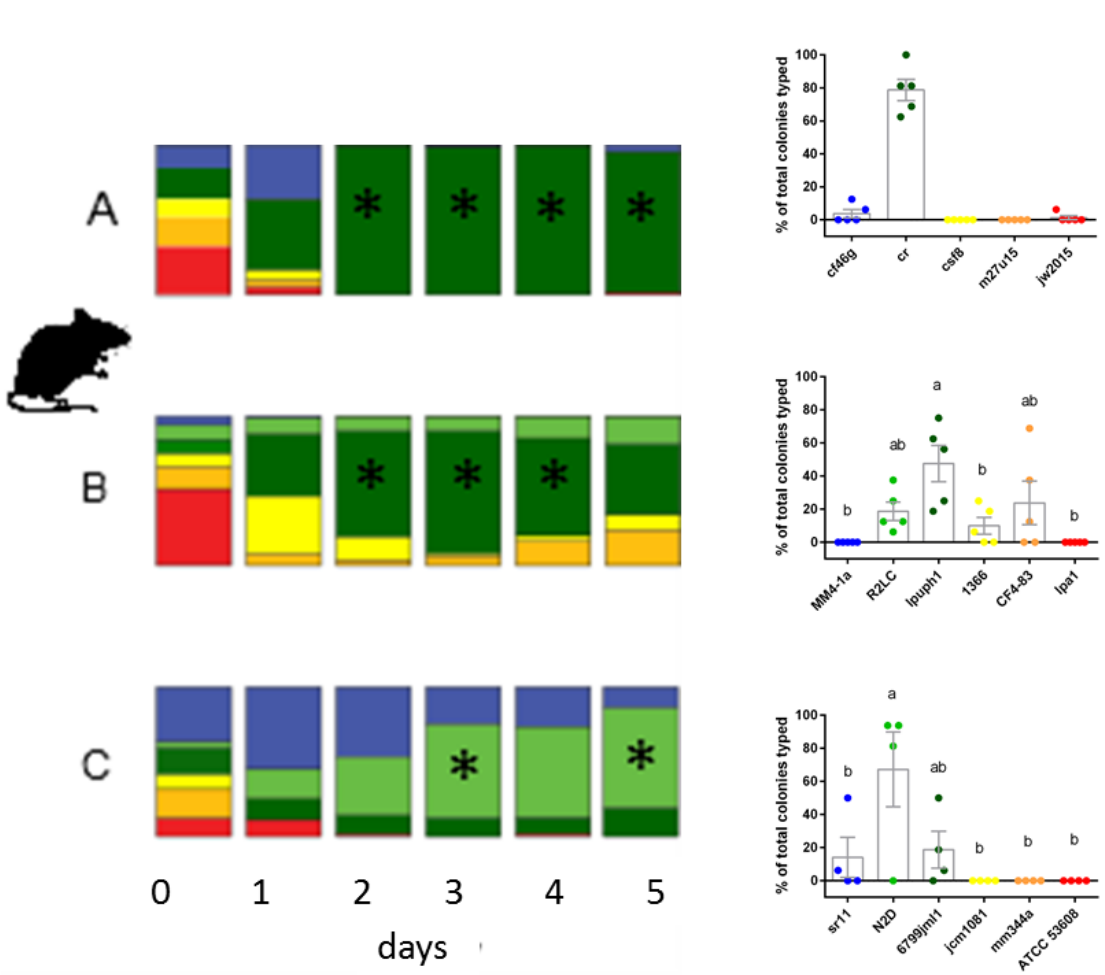

Figure 2.4 Stacked bar plots showing the relative abundance of *L. reuteri*

Stacked bar plots showing the relative abundance of *L. reuteri* strains in the inocula and feces of mice, pigs and humans and cloacal swabs of chickens at baseline and during days 1 to 5 after oral administration of 3 different inocula (A, B and C). An asterisk denotes when a strain from host-specific lineage is significantly higher (p<0.05) than all strains from other lineages in the native host. A white star denotes when the percentage of poultry-VI and the human-VI strains is not significantly different (p<0.05) in chickens. A triangle is shown when a strain become significantly enriched in a non-native host. Adjacent bar graphs show the mean and SE of the relative strain abundance of each strain from the colonies typed at day 5. Individual data points represent the percent colonies typed in each animal or human volunteers. Groups labeled with different letters are significantly different (p<0.05). Statistical significance was determined by one-way ANOVA, α = 0.05.

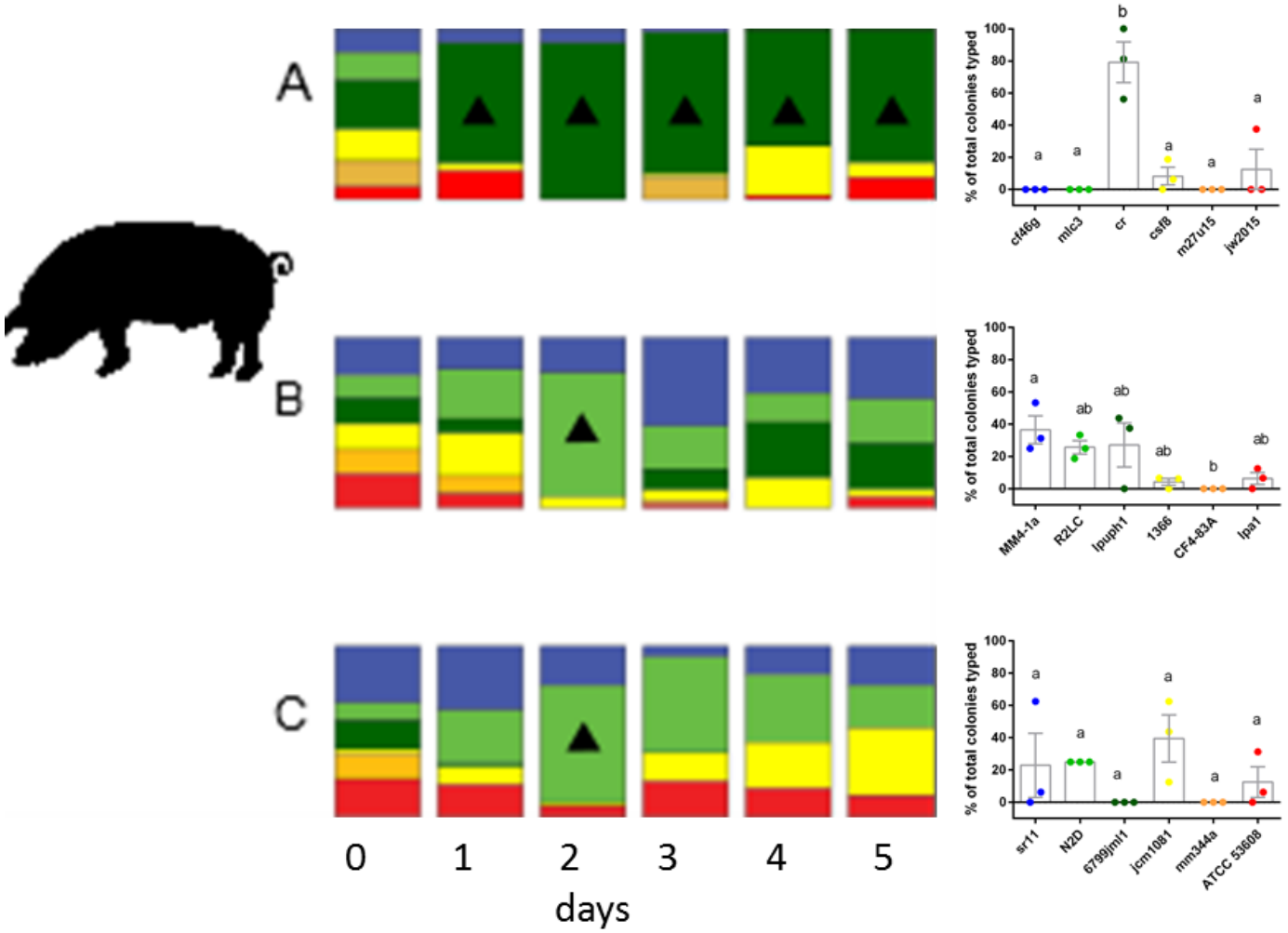

Figure 2.4 (cont). Stacked bar plots showing the relative abundance of *L. reuteri*

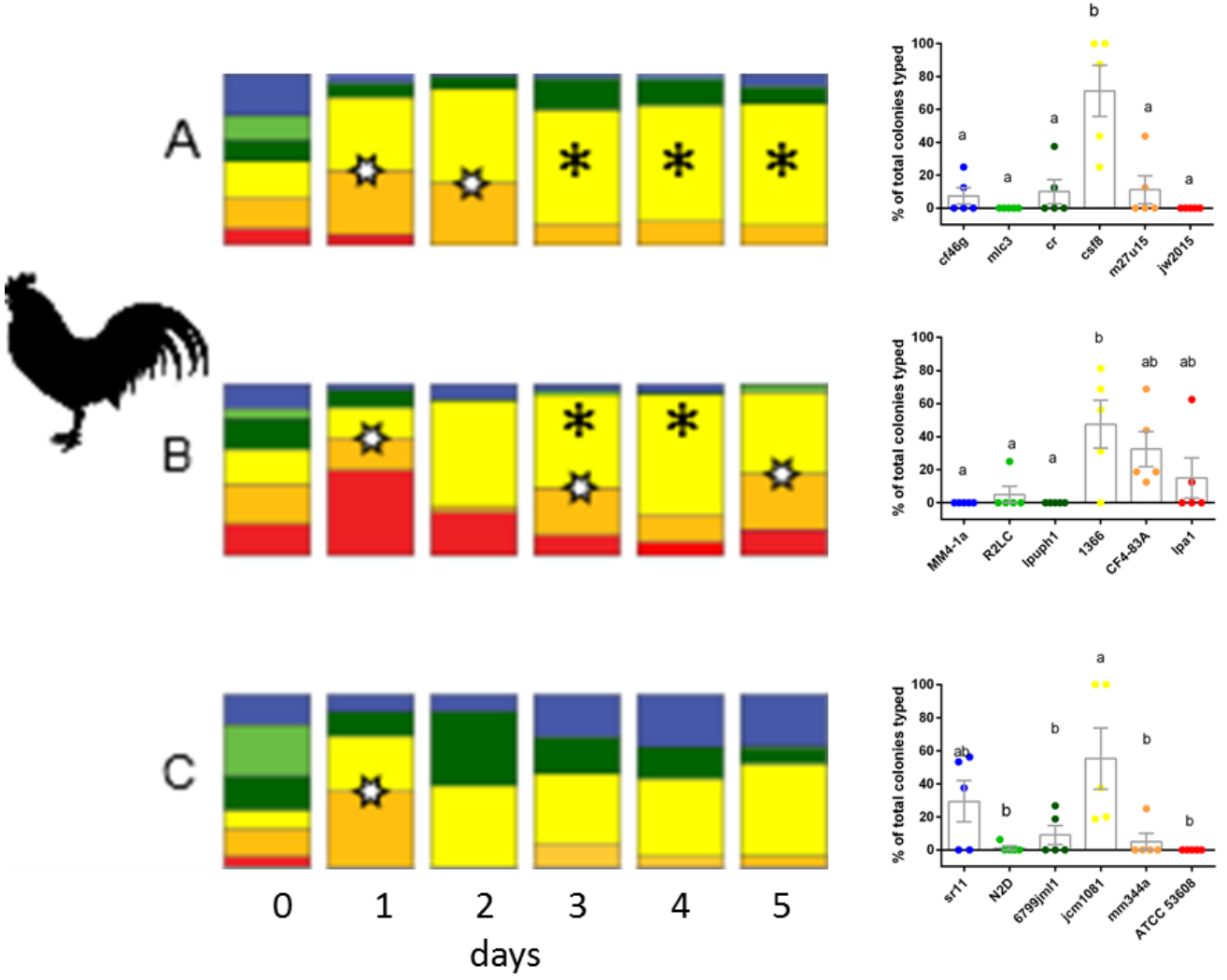

Figure 2.4 (cont). Stacked bar plots showing the relative abundance of *L. reuteri*

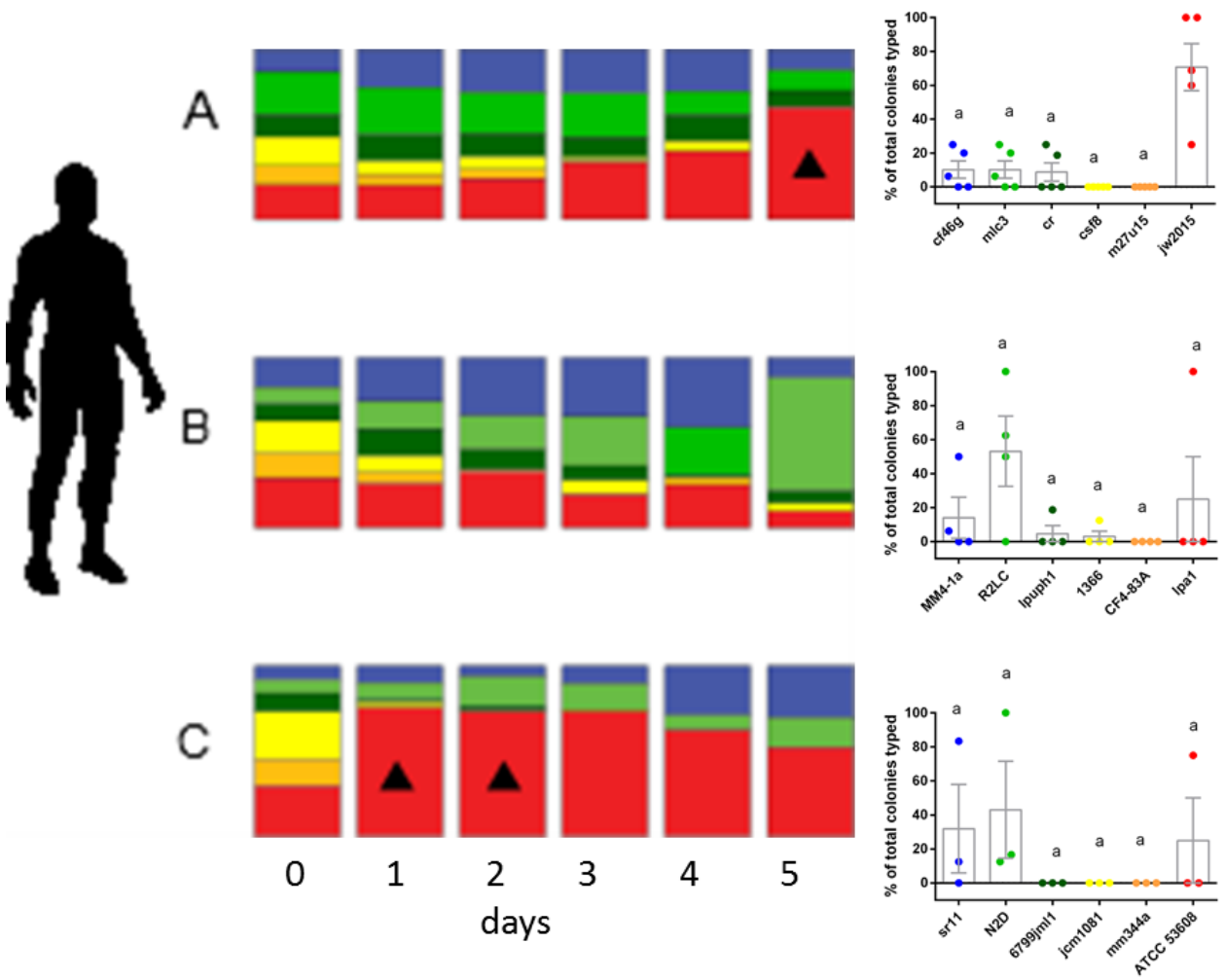

Figure 2.4 (cont). Stacked bar plots showing the relative abundance of *L. reuteri*

2.3.5 The pig host

Contrary to results in mice and chickens, strains of pig origin (porcine-VI) did not become significantly enriched (p<0.05) in pigs at any point during the experimental period (Fig. 2.4). Although some rodent isolates became significantly enriched, this trend was not consistent across inocula, ruling out a competitive advantage of rodent isolates. For example, the strain CR from inoculum A was found in significantly higher amounts (p<0.05) from days 2 to 5 but the strains lpuph1 (inoculum B) and N2D (inoculum C) were only significantly increased (p<0.05) at day 2. Overall, these results indicate that porcine strains do not possess a competitive advantage in their original host.

2.3.6 The human host

Human-II strains were present in the feces of human volunteers during the 5 day post-inoculation period (Fig 2.4) but were not significantly enriched. The only strains reaching significant enrichment (p<0.05) were of porcine origin, however a competitive advantage of these strains can be ruled out as the trend is not consistent across inocula and only observed for one or two days. For example, the porcine strain jw2015 was enriched only at day 5 in volunteers that consumed inoculum A and the strain ATCC 53508 on days 1 and 2 in volunteers that consumed inoculum C. Notably, human-VI strains were essentially outcompeted in the human host. These findings indicate that *L. reuteri* strains originating from humans, regardless of their lineage, did not show elevated levels of colonization in the human gut.

2.3.7 Evolution and genome characteristics of lineage-VI strains

To gain a deeper understanding of the evolution of lineage-VI strains, we reconstructed the ancestral states within the *L. reuteri* phylogeny (Fig. 2.5).This analysis revealed that rodent lineages date back as far as 2 million years, predating all other lineages by at least 800,000 years. The human lineage-II emerged 1.5 million years ago from a rodent ancestor (Event 1). A host

switch approximately 1 million years ago resulted in the emergence of the poultry lineage-VI and porcine lineage-IV (Event 2). The porcine lineage-V appeared much later 96,000 years ago (Event 3). Most importantly, all isolates of lineage-VI share a common ancestor associated with poultry, with the human isolates emerging latest, less than 61,000 years ago (Event 4) (Fig. 2.5).

Next we determined the genomic characteristics of lineage-VI strains by analyzing the genomes of three strains originated from chicken (1366, CSF8 and JCM108) and four strains of human origin (SD2112, MM34-4A, M27U15 and CF483A). As shown in Fig. 2.6 all seven analyzed lineage-VI strains shared 1433 predicted orthologous genes. Beyond this core genome, poultry-VI strains possessed an open pangenome with large number of strain specific genes (197 genes in 1366, 215 in CSF8 and 484 in JCM1081) and an average nucleotide identity (ANI) between 98.77% and 99.06%. This is in stark contrast to human-VI strains which showed very few unique genes and essentially a closed pangenome (between 3 - 6 genes per strain) and an ANI between 99.92% and 99.99%. These findings are in line with the tight clustering of human-IV strains in the phylogenetic tree shown in Fig. 2.1.

We then sought to identify genes specific to (not present in other *L. reuteri* genomes) and conserved in the genomes of all seven strains in the lineage-VI. We found only 3 genes which encoded an aspartate racemase (EC 5.1.1.13) and two transcriptional regulators of the XRE and DeoR families. Next we identified 28 genes that were conserved in the poultry-VI and absent in the genomes of human-VI strains. Of those 10 were annotated as hypothetical proteins, 4 as phage-related, three as c-di-GMP activations via the GGDEF-EAL transduction system involved in biofilm formation (Gjermansen, Ragas and Tolker-Nielsen 2006), and one as a transcriptional regulator of the HxlR family. The remaining encoded proteins involved in vitamin biosynthesis or sugar transporters/transferases. Notably, none of these genes appears to be exclusive to poultry-VI strains when compared to other *L. reuteri* genomes (Table 2.3)

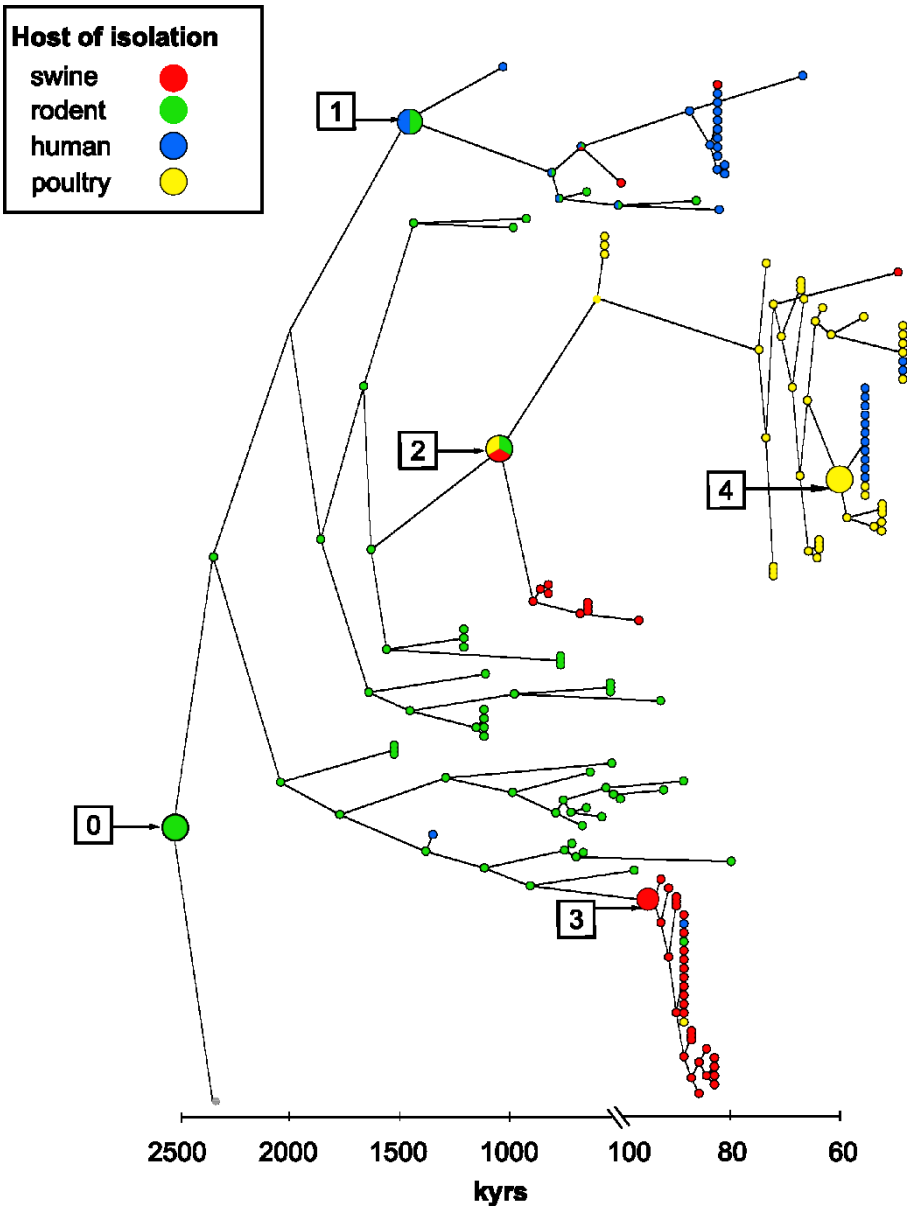

Figure 2.5 Ancestral state analysis

Inferred evolutionary history of *L. reuteri*-host associations. Ancestral states were inferred on bacterial phylogeny modified from a previous study (Oh et al. 2010). The tree is a maximum likelihood reconstruction of a concatenated set of 7 single-copy genes from 116 strains. Colors represent host state on the tips of the tree and inferred states on ancestral nodes. Equivocal ancestral states are represented by mixed colors in the circle. Four host switching events were highlighted as enlarged circles (labeled 1–4). The time scale is in scale of thousand years (kyrs) estimated using Bayesian phylogenetic analyses.

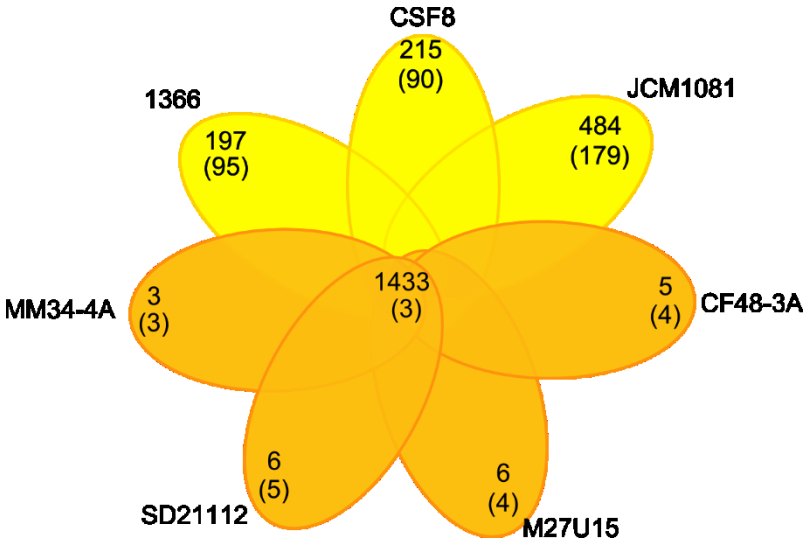

Figure 2.6 Core and strain-specific gene content of *L. reuteri* lineage-Vi strains

Each oval represents the genomes of poultry-VI (yellow) and human-VI (orange) strains. The core gene set is indicated by the number in the center and below in parenthesis is number of unique lineage-VI genes when compared with other *L. reuteri* strains. The outer values in the ovals indicate the number of genes unique to each strain and shown below in parentheses are the numbers of strain-specific genes after subtracting genes found in other *L. reuteri* genomes

Next we examined whether human-VI strains possessed genes not present in poultry-VI strains. Of the 190 proteins identified through this process, 92 were annotated as hypothetical proteins, and 61 as transposases, endonucleases or phage related. Genes with functional annotation that did not fall in these categories are listed in Table 3. Interestingly, 13 of the genes are co-localized in a putatively horizontally-acquired (flanked by transposase genes and 10% higher GC content than the genome average) gene cluster that encodes membrane glycosyl transferases and transmembrane transporters predicted to be involved in capsular polysaccharide biosynthesis. Another interesting human-VI-specific gene included a tetracycline resistance cassette, encoded in plasmid pLR581 previously described for strain *L. reuteri* SD2112 (Rosander, Connolly and Roos 2008). Human-VI strains also harbor three additional plasmids encoding lincomycin resistance (pLR585), cadmium resistance (pLR584), and uncharacterized functions (pLR580). Not all the genes encoded in these plasmids are exclusive to human-VI

strains. However, the replication proteins are conserved in all of the human-VI genomes surveyed (Table 2.3) and the presence of these plasmids was confirmed via extraction and agarose gel visualization (data not shown).

Table 2.3 Genes-specific to Poultry-VI and Human-VI strains

Specific in poultry-VI		Specific in human-VI	
ID[a]	Gene name or function	ID[a]	Gene name or function
20805	GGDEF c-di-GMP synthetase	***64061***	*dTDP-glucose 4,6-dehydratase /epimerase*
20806	EAL c-di-GMP phosphodiesterase	***64063***	*CDP-glycerol phosphotransferase*
20821	Cytochrome Heme/Steroid domain	***64064***	*Glycosyltransferase*
21815	Butanediol dehydrogenase	***64065***	*Wzx-flippase*
21816	Sugar phosphate permease	***64066***	*Transmembrane protein*
21916	UDP-glycosyltransferase	***64067***	*Glycosyltransferase*
22288	Xanthine/uracil /vitC permease	***64068***	*Glycosyltransferase*
22433	Thiamine phosphate synthase	***64069***	*Glycosyltransferase*
22434	Hydroxymethylpyrimidine kinase	***64070***	*1,6-galactosyltransferase*
22959	HxlR transcriptional regulator	***64071***	*Glycosyltransferase*
22963	Sugar transferase LPS biosynthesis	***64072***	*Glycosyltransferase*
23007	Hydroxyethylthiazole kinase	64073	*Glycosyltransferase*
		64074	*Nucleoside-diphosphate-sugar epimerase*
		64355	O-acyltransferase
		64358	Glycosyltransferase
		64361	UDP-galactofuranosyltransferase
		64432	SNF2 family helicase
		64483	ABC transporter ATP-binding protein
		64484	ABC transporter
		64485	LytTR transcriptional regulator
		64759	Glucansucrase
		65314	PglZ alkaline-phosphatase
		65474	Lantibiotic protection ABC transporter

Table 2.3 (cont)

65587	MarR transcriptional regulator
66329	CAAX amino protease (plasmid pLR585)
66330	MerR transcriptional regulator (pLR585)
66332	Replication protein (pLR585)
66333	Replication associated protein (pLR585)
66334	Replication associated protein (pLR585)
66336	Replication protein (pLR580)
66348	Tetracycline resistance protein Tet (pLR581)
66357	Initiator RepB protein (pLR581)
66364	Replication rep protein (pLR584)
66370	Lysophospholipase family protein (pLR584)
66374	Transcriptional repressor CopY (pLR584)

[a]Gene ID as assigned by JGI to *L. reuteri* SD2112 and *L. reuteri* JCM1081 for the genes specific to poultry-VI human-VI, respectively. Genes IDs in bold are exclusive to human-VI strains and genes in are italics are co-localized at the Wzx-dependent capsular polysaccharide synthesis cluster. For non-chromosomal genes the name of the harboring plasmids is shown in parenthesis

2.4 Discussion

In this study, we devised an experimental approach that allowed us to directly assess the competitive interactions among *L. reuteri* strains in the gastrointestinal tracts of different vertebrate hosts. By testing for the relative enrichment of a specific strain, the assay directly compares the ability of strains to propagate under the ecological conditions of the gut. Since population growth directly relates to ecological fitness of microbes (Garcia and Gerardo 2014), an enrichment of strains from a particular phylogenetic lineage, in our models, does provide direct evidence for adaptedness. An interpretation of the findings can therefore be used to infer the evolutionary history of a phylogenetic lineage and the underlying mechanism that drove its diversification.

We tested this approach and confirmed host adaption of rodent isolates to the mouse gut. As shown previously (Oh *et al.* 2010; Frese *et al.* 2011), colonization phenotypes were in agreement with phylogeny as both mouse and rat strains show similar ecological performance in mice. This suggests that rodent lineages are not evolutionarily confined to a specific host species but are adapted to specific gastrointestinal conditions that are expected to be very similar in mice, rats and possibly other rodents (Oh *et al.* 2010). Similar trends have been observed for the bumble bee symbiont *Snodgrassella alvi* which appears to have adapted to the host genera (*Bombus*) and not the species (Kwong and Moran 2015). Although much remains unknown regarding the dynamic evolutionary patterns and adaptive paths of *L. reuteri* strains within rodents, our findings in mice indicate that competition experiments with standardized mixtures of strains are appropriate to make accurate assessments of host adaption, and therefore allow insight on the evolutionary outcomes of host-microbe interrelationships.

2.4.1 Lineage VI strains, even if isolated from humans, show elevated fitness in chicken

In agreement with previous studies (Oh *et al.* 2010; Spinler *et al.* 2014), the genome-wide phylogenetic analysis of *L. reuteri* showed that strains isolated from poultry cluster together with a subset of human isolates into one cohesive phylogenetic lineage (lineage-VI). Although we have previously speculated about the natural history of this lineage (Oh *et al.* 2010), interpretation of phylogenies alone can lead to incorrect conclusions (Moran and Sloan 2015). Our experiments here, now demonstrate that poultry-VI strains are host adapted to chickens and that human-VI strains also performed well in this host, while showing an extremely low performance in humans. Accordingly, the genomic analysis revealed that human-VI isolates are likely to possess all the colonization factors necessary to colonize the chicken gut, as only very few genes were present in poultry-VI strains were absent from human-VI strains (Table 3). Both poultry and human lineage-VI strains possess a lineage-specific putative regulatory system, suggesting an important role of environmental sensing in the colonization of the chicken gastrointestinal tract. In this

regard, a single regulatory gene has been shown to alter host-specificity in *Vibrio fischeri* (Mandel *et al.* 2009) and rodent-specific two component systems are known to regulate biofilm formation of *L. reuteri* in mice (Frese *et al.* 2011, 2013). These findings indicate that human-VI isolates, alike poultry-VI isolates, share an evolutionary history with poultry. Accordingly, the immediate ancestral node of human-VI strains was inferred to be associated with poultry (Fig 5).

The above-mentioned findings beg the question of why lineage-VI strains have been isolated from humans if the lineage has maintained stringent evolutionary ties with poultry and are host-adapted? One possibility is that *L. reuteri* switched from poultry to humans and became permanently associated with this new host. However, in our human experiment, human-VI strains were completely outcompeted, thus ruling out an adaptation to the human gastrointestinal tract. In addition, the majority of the human-VI isolates originate from extra-intestinal sources (breast milk, vagina, mouth) for which the species *L. reuteri* has not been described as a significant member (Ravel *et al.* 2011; Jost *et al.* 2013), which is contrary to poultry where *L. reuteri* is autochthonous and an abundant member of the gut microbiota (Abbas Hilmi *et al.* 2007). Based on these considerations, we propose that specific strains from lineage VI can become transiently associated with humans. Microbial exchange between avian and humans is not only possible but frequent, as demonstrated by 2.5 million cases per year of food borne illnesses in the United States that arise from the transmission of pathogens from poultry, meat, and eggs in the United States (Painter *et al.* 2013). Interestingly, our genomic analysis revealed that poultry-VI strains possess a large and adaptable pangenome, while human-VI strains show very little genomic variation. These findings suggest that essentially one single clone (< 375 SNPs when compared to *L. reuteri* SD2112) amongst the lineage-VI has been repeatedly isolated from humans. Similar phenomena have been observed for globally spread monomorphic pathogens (Achtman 2012), such as *Yersinia pestis* in which the acquisition of a few horizontally acquired traits was sufficient to enable switching hosts (Achtman *et al.* 1999). Under this scenario, and given the high number

of human-VI specific mobile genetic elements found in the genomes of human-VI strains, it is possible that this clone acquired specific traits that allowed a temporary migration to humans. The Wzx-dependent capsular polysaccharide biosynthetic gene cluster and the plasmid-encoded antibiotic resistance cassette, which are both likely to be horizontally acquired, might represent key traits that allow transfer to humans. The ability of these strains to grow in the presence of tetracycline was confirmed experimentally (data not shown) and capsular molecules can induce or suppress host immune responses (Comstock and Kasper 2006; Willis and Whitfield 2013). Under this perspective, it is tempting to speculate about a scenario in which a specific *L. reuteri* clone was able evade the immune system and temporality colonize the human host, presumably after a course of antibiotics.

2.4.2 Human and porcine isolates do not show elevated ecological fitness in their respective hosts

Human-II strains cluster separately from all other *L. reuteri* strains. This lineage is both remarkably homogeneous and specific to humans (Oh *et al.* 2010; Walter, Britton and Roos 2011). However, our experiment did not provide sufficient evidence of adaptation of these strains to humans, even though they persisted more than human-VI strains. This finding could suggest that the evolution of the human-II lineage was not driven by specialized adaptations to the human gut. Another possible explanation is that these isolates have a non-human niche (i.e., environmental, food, other hosts). However, the phylogenetic cohesiveness of these strains argues strongly against this scenario. It is widely documented that food and plant-derived lactobacilli strains are commonly isolated from human fecal samples but these strains, unlike *L. reuteri* human-II strains, are neither phenotypically nor phylogenetically related (Siezen *et al.* 2010; Smokvina *et al.* 2013; Martino *et al.* 2016). We cannot rule out that the human resident microbiota could have prevented the experimental *L. reuteri* strains from becoming established. Although recent work provided evidence that a gut microbe can engraft in the human gut if an

open niche is available (Maldonado-Gómez *et al.* 2016). In this respect, it is important to point out that *L. reuteri* was commonly detected as a member of the human gut microbiota according to studies conducted between 1950 and 1960 (Reuter 2001), while it is rarely detected among the human gut microbiota today (Walter 2008; Qin *et al.* 2010; Martínez, Muller and Walter 2013). This suggests that *L. reuteri* might have been displaced as a dominant member of the human gut microbiota due to environmental changes (e.g., antibiotic usage, hygiene and dietary practices) associated with modern lifestyles (Walter, Britton and Roos 2011) as it has been proposed for other members of the microbiota (Blaser and Falkow 2009). Although speculative, this idea is supported by a recent study in which *L. reuteri* was found to be a dominant member of the microbiota of rural Papua New Guineans (Martínez *et al.* 2015). Under this perspective, the absence of the niche in which human-II *L. reuteri* evolved, would have prevented these strains from becoming enriched in our human experiment. Further work will be required to resolve outstanding questions regarding the evolution of *L. reuteri* with humans.

Contrary to the situation in modern humans, *L. reuteri* is a core member and one of the most abundant *Lactobacillus* species of the pig's microbiota (Leser *et al.* 2002), suggesting *L. reuteri* is symbiont of pigs. However, a recent genome-wide comparative study did not find any host-specific genomic signatures among porcine isolates that cluster into two pig-confined phylogenetic clades (Oh *et al.* 2010; Wegmann *et al.* 2015). This finding suggests that clustering of these strains could be driven by neutral evolution or ecological factors not directly related to the porcine host. Our data agrees with this notion as we found no evidence for host adaptation in pigs. Nevertheless, it is important to point out that our pig model is not exempt of limitations that could explain these observations. For example it is conceivable that complex interactions with the microbiota (both cooperative and antagonistic) might underlie the adaptation of *L. reuteri* to pigs. *L. reuteri* is one member of a multi-species biofilm that forms on the squamous keratinized lining of the pig's pars esophageal tissue (61). In this sense, the germ-free pig model might fail to

recapitulate adaptive interactions between *L. reuteri* and co-existing members of the biofilms, such as *L. amylovorus* and *L. johnsonii*, which are the dominant *Lactobacillus* species in pigs (Leser *et al.* 2002). Overall, our findings suggest a direct co-evolution of *L. reuteri* with rodents and chicken (potentially as a primary colonizer in the biofilms), while in pigs, the species may evolve in a tripartite inter-relationship with the host and other microbes.

Another important factor to consider is that dietary glycans can have a direct effect in the composition of the pig's microbiota, including the abundance of lactobacilli (Frese *et al.* 2015). The infant formula fed to piglets contained galactooligosaccharides (GOS) that are utilized by almost all *L. reuteri* strains independent of host origin (Goin 2010). It is possible that porcine *L. reuteri* strains have evolved to utilize oligosaccharides in the milk of pigs and that this adaptation resulted in the host-confined phylogenetic clusters. A formula in which these carbohydrates are replaced by the non-selective GOS would have rendered strain-specific differences in the ability to utilize milk carbohydrates insignificant, potentially removing the ecological advantage of porcine isolates. Additional animal experiments will ultimately be necessary to derive clear conclusions on the existence of host adaptation of *L. reuteri* to pigs and the mechanisms by which the porcine-specific lineages arose.

2.4.3 Limitations of this study

It is extremely difficult to experimentally replicate the ecological conditions under which bacteria evolve, which are often dynamic and subject to change. The animal host is an excellent replication of the natural habitat of a gut symbiont, but as described above, both germ-free and conventional models have limitations. Germ-free models fail to replicate the interrelationships between members of the community, which might be especially relevant in the evolution of *L. reuteri* as a component of biofilms. On the other hand, in hosts with a conventional microbiota, such as the humans in our study, the niche might already be occupied by more resilient taxa, thus

preventing the establishment of external strains. Future studies should be devoted to apply this experimental system to more refined models such as gnotobiotic animals, especially containing species that co-exist with *L. reuteri* in natural settings.

2.5 Conclusion

Results from this study contribute to our understanding about the evolutionary history of the gut symbiont *L. reuteri*, which is now the first vertebrate gut symbiont for which specific adaptation has been experimentally proven in different hosts. This finding suggests that long periods of strict host-association is required for functional adaptation. This work also expands our knowledge about the various lifestyles and the array of selective pressures shaping the evolution of *Lactobacillus* species. For example, the adapted lifestyle of *L. reuteri* sharply contrasts with that of the generalists *L. plantarum* (Martino *et al.* 2016), *L. paracasei* (Smokvina *et al.* 2013) and *L. rhamnosus* (Douillard *et al.* 2013). This aspect can be particularly important in the selection of probiotics, as functional attributes can be directly related to the evolution of particular strains and the nature of the symbiotic relationship maintained with different hosts (Spinler *et al.* 2014). Survival and persistence in the digestive system might also be a desirable trait for some probiotic applications. Therefore, our findings also provide an ecological and evolutionary basis for the selection of strains for probiotic applications in different hosts.

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3. Chapter three: A rodent-strain specific two-component system is involved in biofilm formation of *Lactobacillus reuteri* 100-23.

3.1 Introduction

The ability of bacteria to evolve and adapt to new habitats is often associated with changes in gene content, genetic regulation and consequently in their functional capacity (Altermann, 2012). These effects are imposed by constraints encountered in the ecosystems they inhabit and adapt to (Alm, Huang and Arkin 2006; Toft and Andersson 2010). The bacterial species *Lactobacillus reuteri* inhabits the gastrointestinal tract (GI) of vertebrate animals (Tannock 1992; Leser *et al.* 2002; Abbas Hilmi *et al.* 2007) and shares an evolutionary history with pigs, rodents, poultry and humans, that resulted in the emergence of phylogenetic lineages associated with host origin (Oh *et al.* 2010; Walter, Britton and Roos 2011; Duar *et al.* 2017 (Chapter 2)). The evolution of *L. reuteri* with rodents was adaptive and resulted in host specificity (Oh *et al.* 2010; Frese *et al*. 2013; Duar *et al.* 2017 (Chapter 2)). *L. reuteri* are dominant members of the murine microbiota and maintain high populations throughout the life of the animal, in part facilitated by their ability to form biofilms in the mouse forestomach, a trait that is exclusive to rodent isolates (Frese *et al.* 2013). The genomes of rodent strains encode a series of functions that reflect adaptations to the conditions in the mouse forestomach and are likely to be determinants of host-specificity. These factors include large adhesin-like surface proteins, SecA2-SecY2 accessory secretion systems, a urease cluster and two-component regulatory systems (Frese *et al.* 2011). Most of these genes are exclusively found in the genomes of rodent isolates and are rarely found or absent in strains from other hosts (Frese *et al.* 2011). Studies with loss-of-function mutants have demonstrated that these genes are elemental to the ecological success of *L. reuteri* in the mouse GI tract (Frese *et al.* 2011, 2013; Krumbeck *et al.* 2016). The urease cluster mediates resistance to acid conditions encountered in the forestomach (Wilson *et al.* 2014; Krumbeck *et al.* 2016). Selective epithelial adhesion and subsequent biofilm formation, are initiated through a rodent-specific surface adhesin serine-rich protein with a devoted SecA2-SecY2 transport system (Frese *et al.* 2013).

Biofilms are sessile layers of bacteria encased in an extracellular matrix composed of exopolysaccharides, proteins and nucleic acids (Branda *et al.* 2005). Biofilm formation is a highly coordinated mechanism often driven by a response to changing environmental conditions (Abee *et al.* 2011), and genes involved in the process are often transcriptionally regulated (Davey and O'toole 2000). In gram positive bacteria, transcriptional regulation of biofilm formation is commonly mediated by two-component regulatory systems (TCS) which typically consist of a membrane-bound histidine kinase (HK) sensor and a cytoplasmic response regulator (RR) (Monedero, Revilla-Guarinos and Zúñiga 2017). HKs respond to extracellular cues by transferring a phosphate group from a conserved histidine residue to an also conserved aspartate residue in a cognate RR. Phosphorylation of the RR subsequently activates the transcriptional output of the pathway (Capra and Laub 2012). TCSs are involved in the regulation and development of biofilm formation of a wide number of bacterial species and a variety of habitats and conditions (Li *et al.* 2002; Zhang *et al.* 2004; Mikkelsen, Sivaneson and Filloux 2011; Su and Gänzle 2014; Norsworthy and Visick 2015) but more importantly, a specific two-component sensor kinase can determine host-specificity, as it has been demonstrated for the marine symbiont *Vibrio fisheri* (Mandel *et al.* 2009).

In a previous comparative genomics study two different TCS were identified to be highly specific to rodent strains (Frese *et al.* 2011). One of these systems was characterized by Su and Gänzle (2014) and found to be relevant to biofilm formation in a substrate dependent fashion (Su and Gänzle 2014). The other TCS (TCS_70529-30) encodes an AgrA-family sensor histidine kinase (HK_70529) and a LytTR transcriptional regulator (RR_70530). Inactivation of an ABC-type transporter located downstream of TCS70529-30 impairs biofilm formation *in vivo* (Frese et al. 2013). However, the specific role of TCS70529-30 in biofilm formation of *L. reuteri* remains to be determined. It was therefore the aim of this study to characterize the role of the rodent-specific TCS70529-30 in *L. reuteri* using the rat isolate 100-23 as a model. Single gene knockouts of the individual genes were generated, and the ability of deletion mutants to form biofilms *in vivo* was

determined in ex-germfree mice and compared to the wildtype (WT). To gain further insight into the specific roles of TCS70529-30 in the structural integrity of the biofilm, macroscopic and microstructural differences in the architecture of the biofilm matrix as well as the analysis of the mechanical strength properties, were conducted using *in vitro* techniques.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 3.1. *L reuteri* strains used for this study were derived from the wild-type strain 100-23. *L. reuteri* strains were grown in modified MRS medium (mMRS) supplemented with 10 g/l maltose and 5 g/l fructose at 37 °C, unless otherwise noted. When antibiotic selection was required, 10 mg/l of ampicillin or 5 mg/l of erythromycin were added to the mMRS media. *Escherichia coli* DH5α was used for cloning procedures. Ampicillin (100 mg/l) or erythromycin (500 mg/l) was added to Luria-Bertani media as needed for selection of clones.

Table 3.1 Bacterial strains and plasmids used in this study

Strain or Plasmid	Genotype	Reference
Strains		
Escherichia coli DH5α	Cloning host for pUC19 and PJRS233-derivative plasmids	(Sambrook and W Russell 2001)
Lactobacillus reuteri 100-23	Wildtype	(Wesney and Tannock 1979)
Δ*hk70529*	Deletion of the *hk70529*	This study
Δ*rr70530*	Deletion of the *rr70530*	This study
Plasmids		
pUC19	Cloning vector used in *E.coli*; Amp^r	(Yanisch-Perron, Vieira and Messing 1985)
pJRS233	Shuttle vector used in the hosts *E. coli* and *L. reuteri 100-23*; Erm^r	(Perez-Casal *et al.* 1993)

Erm^r, erythromycin resistance gene ; Amp^r , Ampicillin resistance gene

3.2.2 DNA manipulations

Routine molecular biology techniques were performed according to standard procedures (Sambrook and W Russell 2001). Restriction and modifying enzymes (Thermo Scientific FastDigest) were used as recommended by the manufacturer. Plasmid DNA was prepared from *E. coli* using QIAGEN Miniprep kits. Chromosomal DNA and plasmid DNA were isolated from *L. reuteri* as previously described in Oh *et al.* (2010) and Walter *et al.* (2003), respectively.

3.2.3 Generation of *L. reuteri* 100-23 knockout mutants

In-frame knockout *L. reuteri* mutants were generated according to a deletion strategy described previously (Su, Schlicht and Gänzle 2011). Plasmids and primers are listed in Table 2. Briefly, ~1kb upstream and downstream regions of the target genes were amplified by PCR from genomic DNA, and referred to as flanking regions FR-A and FR-B, respectively. Amplicons of FR-A and FR-B were inserted separately into pUC19 vector to generate p*Gene*FR-A and p*Gene*FR-B. Flanking regions were then digested with the corresponding restriction enzymes and ligated into pUC19 using T4 DNA ligase to produce p*Gene*_FR-AB. The ligated fragments were digested from p*Gene*_FR1-4 and inserted into the shuttle vector PJRS233 (Perez-Casal *et al.* 1993) to generate the knockout plasmid p-G*en*e_KO. The resulting knockout plasmid was purified and used to electrotransform (12. kV/cm, 25 µF capacitance, 400 Ω resistance) 100 µl of competent *L. reuteri* 100-23 cells. Electrotransformed lactobacilli were incubated in 1.0 ml of pre-warmed mMRS at 37°C for 2.5h to allow phenotypic expression and then plated on mMRS agar plates containing erythromycin (10 µg/ml). After incubation for 24 hours, 10 individual colonies carrying the plasmid were transferred into mMRS-erythromycin broth (10 µg/ml) and incubated at 45°C for 80 generations. Following, single-crossover *L. reuteri* mutants were cured by culturing in mMRS broth at 37°C for 100 generations. Antibiotic sensitive double-crossover mutants were identified by replica plating onto mMRS and mMRS- erythromycin agar plates. In-frame deletions

were confirmed by PCR and Sanger sequencing using the primer set gene-KO-F and gene-KO-R (Table 3.2).

Table 3.2 Primers used in this study to generate knockout mutants

Primer name	Sequence 5' to 3'	Features
70529HK_KO1_BamHI	ATT**GGATCC**GCAAACAGTAAACGCCAAAA	Forward primer for 5' flaking sequence of hk70529
70529HK_KO2_SalI	CCGAAT**GTCGAC**TTGTGCTAACGTTAATTGAATCATC	Reverse primer for 5' flaking sequence of hk70529
70529HK_KO3_SalI	CCGAAT**GTCGAC**ACTTGTTTTATTCAGGGAAAGTGAG	Forward primer for 3' flaking sequence of hk70529
70529HK_KO4_HindIII	ATG**AAGCTT**CAAAATTCGTAAGCCTTTCTGC	Reverse primer for 3' flaking sequence of hk70529
70530RR_KO-F	TGGTGGATTTTGATTTAGAAACG	Forward sequencing primer of Δ*hk70529*
70530RR _KO-R	GTGAGTATCCCCATCCTCCA	Reverse sequencing primer of Δ*hk70529*
70530RR_KO1_BamHI	ATT**GGATCC**CGTCCCAAACTGAGATGGAT	Forward primer for 5' flaking sequence of 70530RR
70530RR_KO2_SalI	CCGAAT**GTCGAC**TACTTTTAACATTTTATTCTCACTTTCC	Reverse primer for 5' flaking sequence of 70530RR
70530RR_KO3_SalI	CCGAAT**GTCGAC**CTCGAAAAGGAAAACCACTAACTAC	Forward primer for 3' flaking sequence of 70530RR
70530RR_KO4_HindIII	ATG**AAGCTT**AATCACATGCGCAATCAATG	Reverse primer for 5' flaking sequence of 70530RR
70529RR_KO-F	TGCCGGGTTCAGAAATAAAA	Forward sequencing primer of Δ*rr70530*
70529RR_KO-R	TCCGCTGAAAAAGAATAATGG	Reverse sequencing primer of Δ*rr70530*

Restriction enzyme sites are indicated in **bold**

3.2.4 Mouse experiments

Germ-free Swiss Webster mice were bread and maintained in sterile flexible polyurethane isolators at the University of Nebraska Gnotobiotic Mouse Facility. *L. reuteri* strains (Table 3.1) were tested for production of biofilm according to the protocol described by (Frese *et al.* 2013). Briefly, mice were gavaged 100µl with of a PBS solution containing 10^7 viable *L. reuteri* cells, then moved into biocontainment cages and housed in groups of two. After 48 hours mice were sacrificed and forestomach tissue and contents collected and processed for culture plating and for microscopic analysis following methods by Frese *et al.* (2013). Germ-free status of the ventilator system was confirmed by aerobic and anaerobic nonselective (BHI) culture of fecal pellets of two control mice gavaged with sterile PBS and maintained in the same ventilated rack as the experimental mice. All animal experiments were performed in conformity with protocols #1022 and 1215 approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

3.2.5 *In vitro* biofilm assays

L. reuteri were grown in mMRS supplemented with 1 g/l sucrose (suMRS) and incubated anaerobically (5% CO_2,5% H_2, and 90% N_2) at 37°C for 48h. Three colonies were then grown overnight in 5 ml suMRS broth before sub-culturing in 5 ml of the same media and grown for another 16h. The cultures were then diluted in fresh suMRS to reach a standardized cell suspension of OD_{600} 0.05 and 10 ml of this suspension were transferred to sterile polystyrene tissue culture plates (SARSTEDT®) and incubated statically at 37 °C for 24 hours.

Quantitative determination of biofilm formation was performed by the spectrophotometric method, which measures the total biofilm biomass, including bacterial cells and the biofilm matrix. The optical density at 600 nm (OD_{600}) of planktonic cells was determined in the liquid medium which was carefully removed by aspiration without disturbing the adherent biofilm. Next, loosely adhering cells were removed by three gentle washes with 10 ml of sterile phosphate-buffered

saline (PBS). After the washings, the biofilms were harvested by thoroughly scraping the bottom of the culture plates using disposable sterile cell scraper and resuspended in 10 ml of PBS. Biofilm density was determined by measuring the OD_{600} of this suspension and total numbers of bacteria on the biofilms were determined by the serial dilution plating on suMRS. Each assay was performed at least in triplicate and repeated at least twice.

3.2.6 Mechanical properties of biofilms

Biofilms were grown in tissue culture dishes as described earlier (see *in vitro* biofilm assays). The residual culture media with planktonic cells was remove from the plates by decanting and 5 ml of PBS carefully added to the remaining biofilm using an automatic pipette pump set to the lowest dispensing speed. Plates were then agitated on an orbital shaker at 500 rpm for 30s. PBS containing detached biofilm cells matrix was collected by decantation and the OD_{600} obtained. The process was repeated at increasing intervals of 30, 60, and 300 seconds.

3.2.7 Confocal Microscopy

Visualization and quantification of biofilm formation in the mouse forestomach was conducted by confocal laser scanning microscopy (CLSM) following methods described previously (Frese et al. 2013). Briefly, forestomach tissues were fixed and bacterial cells stained with propidium iodine. Confocal images were obtained by a blinded technician from three random tissue sites. Biofilms were quantified by the red-channel pixel area in images using ImageJ (Schindeli*n et al*. 2015) (Frese et al 2013).

In vitro biofilms for CLSM were grown in ultra-thin, gas-permeable tissue culture plates (LUMOX®). Attached biofilms were stained with Syto-9 and propidium iodine (FilmTracer™, Invitrogen) following manufacturer's instructions. Five random areas of the biofilm on each plate were scanned and obtained with a Olympus IX-81 spinning disk laser scanning microscope using Volocity software (PerkinElmer), resulting in 15 imaged areas per sample. The Syto-9 fluorophore

was excited at 525 nm and the emission band-pass filter used was 550 nm. Excitation of propidium iodine was achieved at 620 nm and emission collected using a 650 nm filter. Simultaneous dual-channel imaging was used to display green and red fluorescence (Hamamatsu EMCCD camera, 60X/1.42 NA). z-stacks were collected at 1.0-µm intervals and the images were compiled in ImageJ using the Volume Viewer 1.31 plugin.

3.2.8 Scanning Electron Microscopy

L. reuteri wild-type and mutant strains were grown in polystyrene tissue culture dishes as previously described. Biofilms formed at the bottom of the plates were fixed in 0.1 M Sorenson's phosphate buffer containing 2.5% EM grade glutaraldehyde for 48h at room temperature. Fixed biofilms were washed three times for 10 min in 0.1 M PBS buffer (pH 7.4). Samples were then dehydrated with a series of 15 min long washings with solutions at increasing ethanol concentrations (50%, 70%, 90%,100% v/v) Hexamethyldisilazane (HMDS) was introduced using gradually increasing HMDS solutions in ethanol as follows: 75% ethanol-25% HMDS (Hexamethyldisilazane), 50% ethanol-50% HMDS, 25% ethanol-75% HMDS, and 100% HMDS. Samples were air dried overnight and then broken down into smaller pieces that were mounted on SEM stubs and sputter coated with Au/Pd (Hummer 6.2 Sputter Coater, Anatech Ltd.). Biofilms were visualized under a scanning electron microscope XL30 (FEI Company) operating at 20 kV. Pictures were acquired using Scandium 5.0.

3.2.9 Statistical analyses

All statistical analyses were carried out using Graph Pad Prism version 6.2. Statistical significance between the percent biofilm formations of the mutants respective to the WT was determined using a two-sided Mann-Whitney U test. Differences among the optical density values were determined using Student’s *t* tests.

3.3 Results

3.3.1 Genetic organization of the two component system 70529-30

A schematic mapof TCS70529-30 and the surrounding genes are shown in Fig. 3.1. The *hk70529*, located at the bp 984225 to 985535 encodes a 436 aa protein containing the Conserved Protein Domain (COG) 2972 sensor histidine kinase YesM. The *rr70530* encodes a protein of 255 aa containing the COG 3279 DNA-binding response regulator of the LytR/AlgR family. Upstream of the *hk70529* are located three small peptides of 32 aa, 53 aa, and 41 aa respectively. Located downstream of *rr70530* is a 52 aa bacteriocin-type signal sequence with a double glycine motif. Immediately after, two genes encode the C-terminal (461 aa) and N-terminal (299 aa) regions of an ABC-type bacteriocin transporter. The N-terminal region contains a double-glycine peptidase domain.

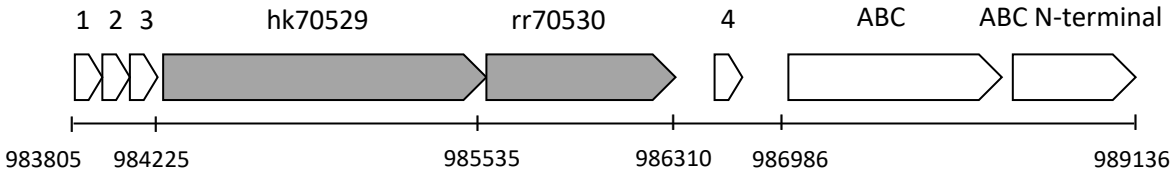


Figure 3.1 Structural organization of the TCS70529-30 genetic locus

Genes were annotated on the IMG ER (http://img.jgi.doe.gov/er). 1, hypothetical protein (99bp); 2, hypothetical protein (162bp); 3 bacteriocin-type signal sequence (126 bp); hk70529, AgrA family, sensor histidine kinase (1311 bp); rr70530, response regulator of the LytR/AlgR family (796 bp); 4, bacteriocin-type signal sequence, contains a double-glycine motif (159 bp); ABC-type bacteriocin transporter (1396 bp); ABC-type bacteriocin transporter, contains an N-terminal double-glycine peptidase domain (720 bp).

3.3.2 Deletion of the *rr70530* but not *hk70529* the resulted in changes in biofilm *in vivo*

To characterize the role of TCS 70529-30, in-frame deletions of the individual genes were generated. Deletion of these genes did not impact the growth of *L. reuteri* 100-23 in mMRS media (Fig. 3.2). The ability of mutant strains to colonize and produce biofilms was determined in mono-associated mice. As shown in Fig. 3.3a, 48 hours postinoculation the biofilm of the hk70529

mutant was equivalent to that of the WT. In contrast, deletion of the *rr70530* resulted in a 50% reduction in biofilm formation. Interestingly, deletion of these genes did not affect the ability of *L. reuteri* to colonize the mouse forestomach, as indicated by cell counts (Fig.3.3b). These data suggest that TCS 70529-30 regulates genes that are important for biofilm formation but not for the survivability of *L. reuteri* in the forestomach.

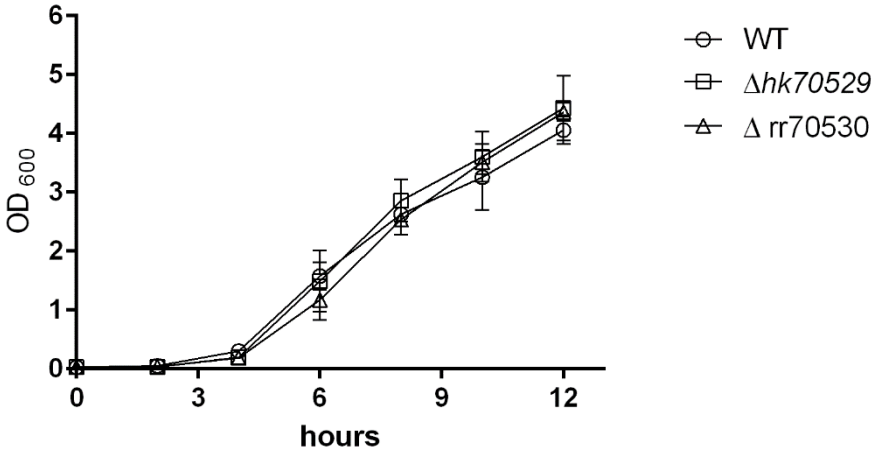


Figure 3.2 Growth curves of the parent strain *L. reuteri* 100 -23 and tΔ*hk70529* and Δ*rr70530*

Strains were grown in MRS medium. Results are shown are the mean ± SD of three independent measurements

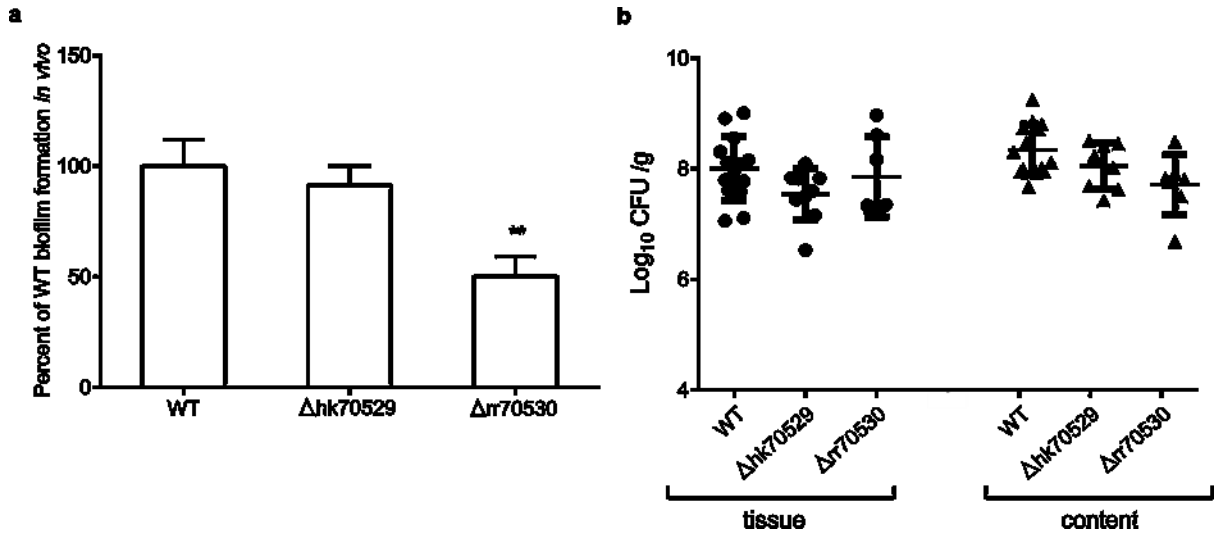


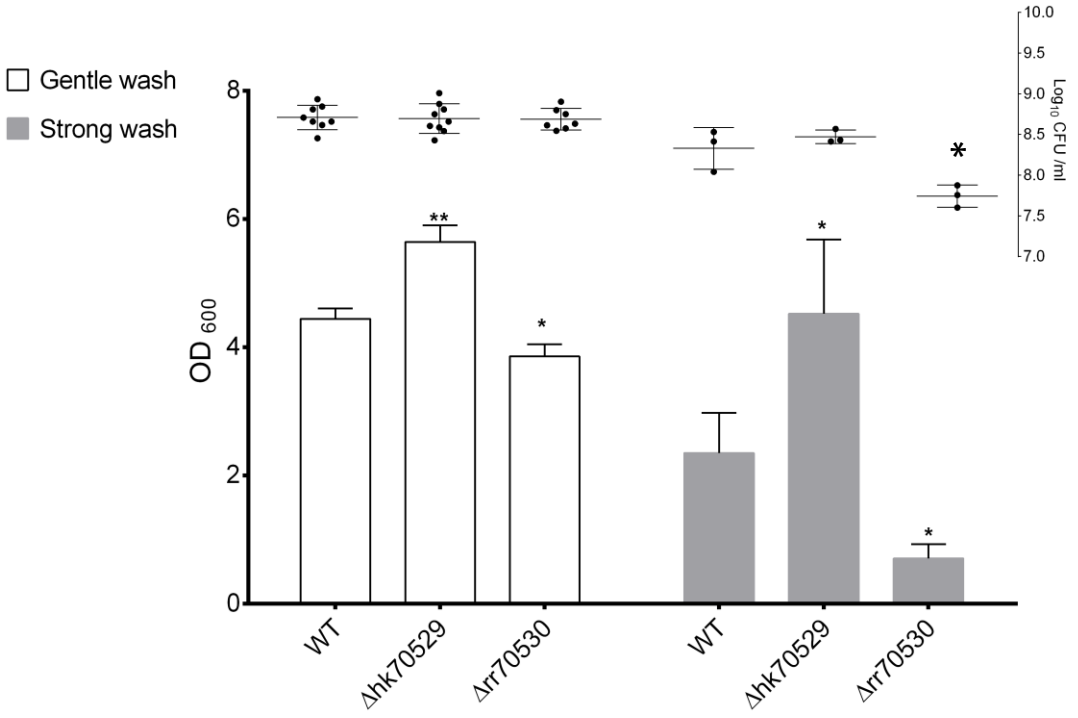
Figure 3.3 Biofilm formation in the cell counts in the mouse forestomach

(a) Quantification of biofilm density relative to biofilm of wild-type strain 100-23 measured as the read pixel area of images obtained by confocal microscopy. Mann Whitney U test ** p<0.01. (b) Cell counts of the wildtype strain and mutants in in the forestomach tissue and content.

3.3.3 Mutation of *rr70530* results in biofilm defects *in vitro*

To investigate the mechanistic basis for the findings observed *in vivo*, the characteristics of the biofilms of mutant strains were examined *in vitro* and compared to the parent strain. Strains were grown on tissue culture plates and the density of biofilms was determined by optical density. After removing residual media and planktonic cells, biofilms were washed gently with PBS. The OD_{600} readings of the remaining biofilms were consistent with *in vivo* findings (Fig. 3.4). Deletion of *rr70530,* but not *hk70529* resulted in significant defects (p<0.05) in biofilm formation. This analysis also revealed that deletion of the *hk70529* increased biofilm formation (Fig. 3.4). In further agreement with the *in vivo* results, no differences in cell numbers contained within the biofilms were observed. Plates were then washed vigorously with PBS, leaving behind only the most strongly adhered cells and their surrounding biofilm matrix. These extra washing steps resulted in a significant reduction (p<0.05) of the biofilm density and the cell counts of the Δr*r70530* respective to the WT, whereas no changes in either component were found in the *hk70529* mutant. As shown in Fig. 3.4, the remaining biofilms of the WT and Δ*rr70530* were

reduced almost by half, while the OD_{600} of the *hk70529* was only reduced from an average OD_{600} 5.6 of 4.5. Together these finding supports the notion that differences in biofilm density of the mutant strains are likely related to structural variations in the biofilm matrix.



The graph represents the turbidity of the biofilms as reflected by their optical density (OD_{600}). Corresponding cell counts are shown. Statistical significant differences from the wildtype are indicated as * $p<0.05$, ** $p<0.01$. Mean values of 3 - 6 independent experiments and SDs are presented.

d

3.3.4 Mutant biofilms exhibit different macroscopic properties and microscale architectures

Visual inspection of the culture plates reavealed differences in the mutant's macroscopic appearance of the biofilms. As shown in Fig. 5a-c, the biofilms produced by the mutant and WT strains differed in their visible morphology. The WT and Δ*hk70529* produced coarse biofilms with defined grooves (Fig. 3.5a and b). In contrast, the biofilm of the *rr70530* mutant was smoother (Fig. 3.5c). A single wash with PBS allowed the visual examination of the internal adhered layers of the biofilm matrix (Fig. 3.5d – f). Removal of loosely adhered layers of the Δ*rr70530* biofilm,

revealed the presence of grooves or channel like structures. However, these appered to be wider and less defined than those of the WT and the Δ*hk70529* (Fig. 3.5f).

To further examine the differences underlying the macroscopic differences of the mutant biofilms, we used CLSM to investigate their microscale architecture. Confocal 3D-rendered images of the biofilms are shown in Fig. 3.5 g-i. Consistent with the macroscopic observations, the overall architecture of the biofilm varied amongst strains. Both the WT and Δ*hk70529* formed compact and well-organized biofilms, but the hk70529 mutant formed a denser biofilm than the parent strain (Fig. 3.5h). On the other hand, the biofilm matrix of the *rr70530* mutant was clearly underdeveloped and bacterial cells appeared attached to the bottom of the plates, forming large intercellular gaps.

Scanning electron macroscopic observation showed that biofilms formed by Δ*hk70529* were more uniform when compared to the WT (Fig. 3.7). On the contrary, large interspersed areas devoid of cells were observed in the biofilms formed by the rr70530 mutant. A closer examination (*x* 10 000 magnification), revealed that in some areas the WT and the hk70529 mutant cells were associated with what appears to be an extracellular polymer. Notably these structures although not completely absent, were less frequently observed in association Δ*rr70530* cells (Fig. 3.7).

Overall, these observations indicate mutations in the TCS70520-30 affect the macroscopic and microscopic characteristics of the biofilms formed by the strain *L. reuteri* 100-23. These differences in the biofilms formation appear to related presence of exopolymers associated with bacterial cells.

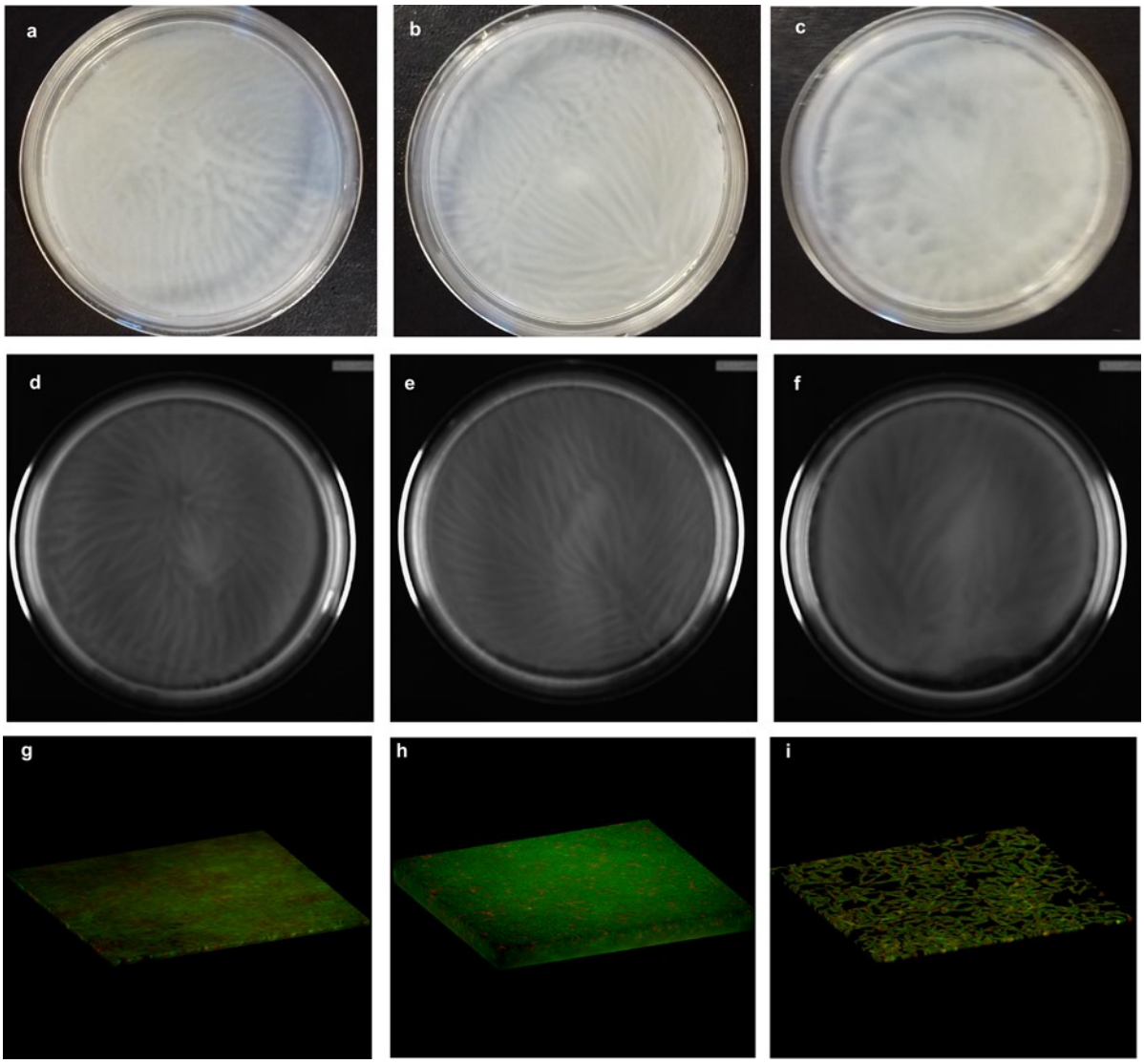


Figure 3.5 Macroscopic and microscopic characteristics of *in vitro* biofilms

L .reuteri 100-23 wild type (a, d and g), *hk70529* mutant (b, e and h) and *rr70530* mutant (c f and i). Top pictures show the biofilm surface after a single PBS wash. Middle pictures show the inner layers of the biofilm after three PBS washes. Bottom pictures are 3D renderings from 1.0-µm z-stacks

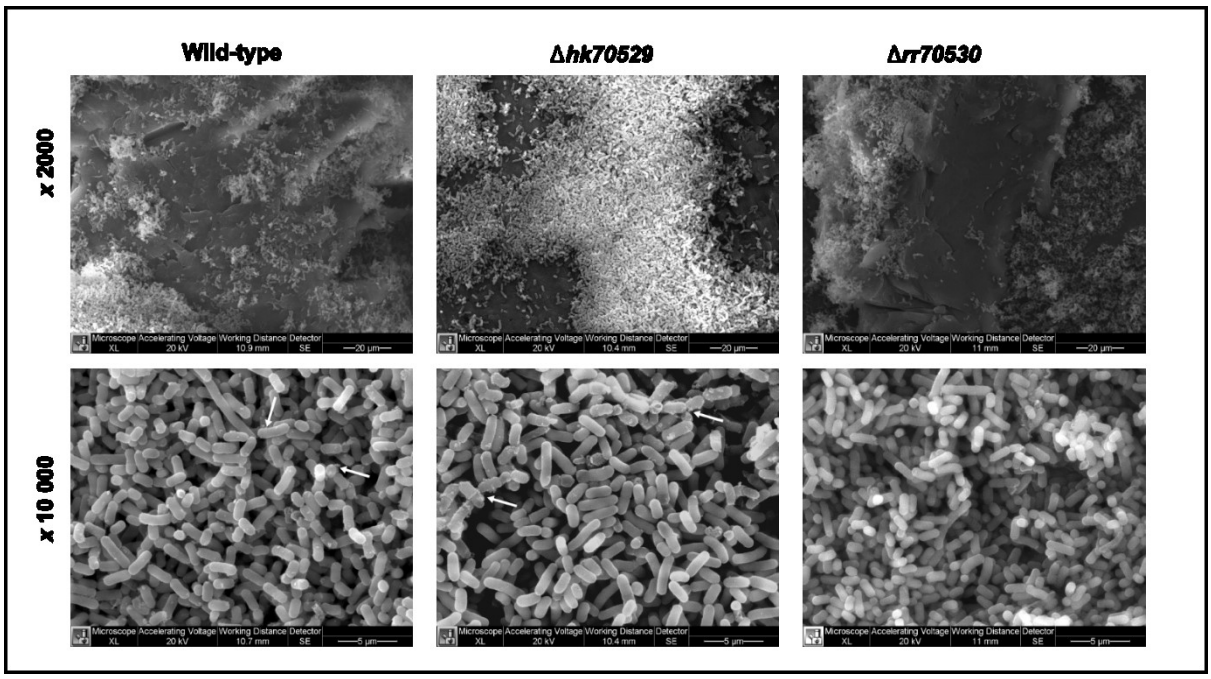


Figure 3.6 SEM micrographs of *L. reuteri* 100-23 wild-type and mutant strains on polystyrene surfaces

Micrographs were taken at two magnifications, top x 2000, bottom x 10 000**.** Arrows indicate possible extracellular polymers.

3.3.5 Matrix architecture is associated with changes in biofilm resistance to sheer stress

To obtain a quantitative and physically informative understanding of the observed differences in matrix architecture, and because the presence of channels has been associated with the hydrodynamic properties of biofilms (Wilking *et al.* 2013), we compared the resistance of the mutant and WT biofilms to hydraulic sheer stress. PBS was added to 24 hour biofilms formed at the bottom of tissue culture plates and resistance to agitation (500 rpm) was measured at increasing time intervals. Resistance was determined relative to the WT as a function of the OD_{600} of the detached biofilm as a consequence of rotational fluid motion. As shown in Fig. 3.8, differences in biofilm architecture correlate with the ability to resist sheer stress. At all the examined time points the denser biofilm produced by the *hk70529* mutant was significantly ($p<0.05$) more resilient than the WT and the *rr70530*. No difference in biofilm strength was found between the WT and the *rr70530* during after agitation for 30s and 60s. However, significant

differences (p<0.05) were observed at longer times of agitation (120s and 420s). Overall, this analysis revealed that differences in biofilm architecture correlate with resistance to sheer stress. This data further support the notion that the TCS70529-30 is likely to regulate genes involved in structural properties of the biofilm matrix.

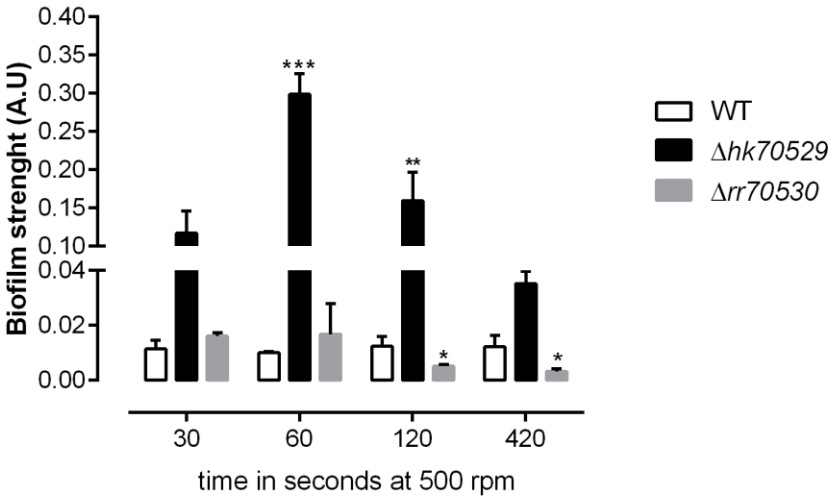


Figure 3.7 Resistance of wildtype and mutant *in vitro* biofilms to sheer stress

Values are provided in arbitrary units (A.U.) calculated as the reciprocal of the percent OD_{600} of the detached mutant biofilm respective to that of the wildtype. Means ± standard errors (error bars) of three biological replicates are shown. Values significantly different from the wildtype (p<0.05) are indicated by an asterisk. Statistical comparisons were done using a one-tailed Student's t test. Significant differences strain are denoted as * p<0.05, ** p<0.01. *** p<0.001

3.4 Discussion

In this study, we assessed the role of a rodent-specific TCS in biofilm formation of the rat isolate *L. reuteri* 100-23. Isogenic deletion mutants where tested for their ability to form biofilms in germ-free mice. Characterization of the physical properties of the biofilms were conducted using a polystyrene plate model. The genetic organization of the TCS70529-30 is that of a typical TCS where the HK and the RR function together (Capra and Laub 2012). However, deletion of the individual genes resulted in disparate phenotypes. Compared to the WT, deletion of the *rr70530* produced a thin and significantly weaker biofilm, while the *hk70529* mutant produced a more robust biofilm, highly adherent and more resistant to mechanical breakage.

Although the canonical TCS consists of a signal relay between a histidine kinase and a cognate response regulator, in reality, many of these transduction systems involve multiple components and multiple phosphorelay cascades and feedback loops servings as “checkpoints”. Moreover, the transcription of one TCS can being controlled by second TCS and certain kinases have shown to interact with non-cognate regulators (Bijlsma and Groisman 2003; Capra and Laub 2012). Biofilm formation in host tissues has proven to be an especially complex process, often involving signal cascades from several TCS. For example, host colonization and biofilm formation of the squid symbiont *Vibrio fischeri* requires input from at least two sensor kinases (Norsworthy and Visick 2015). Similarly, biofilm formation by the opportunistic pathogen *Pseudomonas aeruginosa,* also requires cross-talk between HKs (Goodman *et al.* 2004; Ventre *et al.* 2006). The rodent strain *L. reuteri* 100-23 possesses other TCS in addition to 70529-30 (Frese *et al.* 2011), and at least two of which have been shown to interact at the transcription level (Su and Gänzle 2014). It is therefore quite possible that biofilm formation of *L. reuteri* 100-23 is controlled by a complex signaling network between TCSs and not by one single system.

According to qualitative and quantitative data obtained in this study, alterations in biofilm formation of mutant strains were related to the structure and strength of the biofilm matrix and not to differences in growth rate or cell numbers within the biofilm. The structural organization and

mechanical properties of a biofilm are heavily determined by the composition of its polymeric matrix (Berk et al. 2012; Chew et al. 2014; Persat et al. 2015), which in most bacterial species consists mainly of exopolysaccharides (EPS) proteins, nucleic acids and lipids. EPS mediate cell-to-surface adhesion, cell-cell interactions and serve as scaffolds to the three-dimensional architecture of the biofilm matrix (Flemming and Wingender 2010; Flemming *et al.* 2016). Mutants unable to synthesize or export EPS are typically deficient in biofilm formation (Karatan and Watnick 2009). Inactivation of genes involved in EPS production in *L. reuteri* affects *in vitro* biofilm formation and ecological performance in the murine gut (Walter *et al.* 2008). Functional inactivation of signal transduction systems in biofilm-forming bacteria, has shown to affect biofilm formation through mechanisms related to EPS production (Goodman *et al.* 2004; Ventre *et al.* 2006; Norsworthy and Visick 2015). For example, deletion of the RetS sensor kinase of *Psudomonas aeruginosa* resulted in upregulation of EPS-producing genes, which in turn increased adhesion and biofilm density. Deletion of related HK (*ladS*) repressing the expression of RetS, decreased both biofilm formation and EPS gene expression (Ventre *et al.* 2006). Similarly, *Streptococcus mutans* defective of the TCS *vicRK* involved in the transcription of genes involved in EPS biosynthesis, produce aberrant biofilms (Senadheera *et al.* 2005). Taken together, several studies have shown that the process of biofilm formation is heavily coordinated through TCS and that functional inactivation of these regulatory genes impacts biofilm formation by affecting the transcription of genes involved in EPS production. Although the transcriptional targets of TCS70529-30 are yet unknown, genes involved in EPS production are likely candidates. Since most experimentally characterized LytTR-containing RRs act as transcriptional activators (Nikolskaya and Galperin 2002; Galperin 2008), inactivation of *rr70530* would impair the transcription of genes involved in EPS synthesis, which would easily explain the biofilm phenotype observed for the *rr705030* deficient mutant. Furtheremore, HKs have also been shown to have phosphatase activity, essentially antagonizing the action of RRs (Brooks and Mandel 2016). Given the increased biofilm phenotype of the hk70529 mutant, the most parcimonous explanation

for such obvservation is that *hk70529* acts as a phopatase and not a phosphorylase. Deletion of *hk70529* would then result in upregulation of genes transcriptionally regulated by *rr70530* (e.g EPS biosynthesis) explaining the phenotypes observed. However, additional experiments, such as those decribed by Brookes and Mendel (2016) are thus warranted to fully elucidate the mechanisms by which TCS70529-30 can be involved in EPS biosynthesis.

Besides EPS, extracellular DNA (eDNA) is another important structural component of the biofilm matrix (Sharma-Kuinkel *et al.* 2009; Vilain *et al.* 2009; Harmsen *et al.* 2010; Svensson, Pryjma and Gaynor 2014; Okshevsky, Regina and Meyer 2015). The source of the eDNA, is believed to originate from whole-cell lysis or secretion of outer membrane vesicles containing DNA (Bayles 2007; Sharma-Kuinkel *et al.* 2009; Hobley *et al.* 2015). Interestingly, a LytS system known to play a role in cell lysis in *S. aureus*, was found to be upregulated during biofilm formation of *L. reuteri in vivo.* It would therefore be warranted to characterize the role of TCS70529-30 in the release of eDNA during biofilms formation of *L reuteri.* In this sense, it would also be particularly interesting to determine if the expression of LytS is affected by mutations in TCS70529-30.

In addition to mechanical properties, the structural architecture of the polymeric matrix can have a profound influence in biofilm ecology. Spacing between the bacterial cells effects communication between cells. The organization of the channel network controls the circulation of water and nutrients, as well as the exchange metabolites and signaling molecules that are important for biofilm formation (Wilking *et al.* 2013; Flemming *et al.* 2016)**.** Initial attachment is the most critical step for the establishment of biofilms (Flemming and Wingender 2010). Adhesion of *L. reuteri* to forestomach is believed to be mediated by a rodent-specific large surface protein (Frese *et al.* 2011). Following attachment, a series of adhesive processes link bacteria together into a multilayered three-dimensional structure, ultimately producing a mature biofilm (O'Toole, Kaplan and Kolter 2000; Nobbs, Lamont and Jenkinson 2009). Partial disintegration of the EPS matrix is also an important step in the biofilm cycle. This process is required for dispersal and

colonization of new sites and for the renewal of the bacterial cells within the biofilm (Otto 2013). Mutations in the *arg* quorum-sensing system involved in the detachment and dispersal of *S. aureus* (Boles and Horswill 2008), resulted in increased adherence and biofilm formation (Vuong *et al.* 2000). In this respect, if TCS70529-30 controls biofilm dispersal, deletion of the sensor kinase would halt the detachment process resulting in a denser and more adherent biofilm, explaining the phenotype observed for the hk70529 mutant. Real time microscopy imaging paralleled trascriptome profiling have been proven useful tools to analyse the dynamics of the biofilm development and detachment (Nicholson, Conover and Deora 2012). Similar experiments are thus warrante to determine de involvement of TCS70529-30 in biofilm dispersal.

In conclusion, this work provides empirical evidence of a rodent specific signal transduction system playing a role in biofilm formation of *L. reuteri* 100-23. However, its precise function and the transcriptional genetic targets remain to be identified. Since deletion of individual genes did not affect biomass accumulation but rather exerted effects on biofilm architecture, genes related to the development of the biofilm matrix (e.g. EPS production and release of eDNA) seem to be the most likely candidates, though further experimental evidence will be required to confirm such hypotheses.

The ecological fate of *L. reuteri* depends on its ability to occupy the forestomach niche and persist inside the murine host. It is therefore highly unlikely for the entire process of biofilm formation to be devoted to one single signal transduction system. Instead, the mechanisms governing biofilm are expected to involve complex signaling cascades, likely orchestrated through multiple system transduction systems; as it has been shown for other symbionts (Norsworthy and Visick 2015). Deciphering the role of TCS70530-29 in biofilm formation can foster our understanding of the molecular mechanisms involved in the adaptation of *L. reuteri* to the vertebrate gut. Important challenges for the future studies are therefore, to identify the signals

recognized by the sensor kinase *hk70529* and to determine the target genes of the *rr70530*. Findings from this work open avenues for such studies.

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4. Chapter four: Metabolic cross-feeding between 1,2 propanediol-producing intestinal bacteria and the human isolate *Lactobacillus reuteri* ATCC PTA6475

4.1 Introduction

The human intestine harbors a diverse and complex microbial community composed of trillions of metabolically active bacteria. This community of microbes exists in an intricate mutualistic symbiosis with the host, where microbes provide a number of key functions to the host's well-being and fitness and in return receive secure habitats and nutrients (Bäckhed *et al.* 2005; Clemente *et al.* 2012).

The main energy sources that support the microbial community in the human intestine are nondigestible dietary compounds and endogenous host secretions such as mucin (Koropatkin, Cameron and Martens 2012). As is typical in complex microbial ecosystems, much of the nutrient acquisition in the gut depends on a dynamic network of competitive and cooperative interrelationships amongst members of the community (Coyte, Schluter and Foster 2015). One such interaction, referred to as cross-feeding, occurs when compounds derived from the metabolic output of one bacterium serve as metabolic input for another (Seth and Taga 2014).

Cross-feeding of nutrients is central theme in the fermentative degradation of complex carbohydrates and has a huge impact on the final balance of short chain fatty acids produced in the gut (Flint *et al.* 2012). One important route for the production of butyrate occurs though metabolic cross-feeding interactions between lactate-producing species and lactate-utilizing butyrogenic species (Duncan, Louis and Flint 2004; Rivière *et al.* 2015). Another mechanism involves cross-feeding of fractions released from the partial breakdown of complex polymers (Belenguer *et al.* 2006), such as the degradation of fructans, starch, xylan, and mucin polymers which have been shown to require a series of syntrophic interactions involving different species with complementary enzymatic repertoires and nutritional requirements (Egan *et al.* 2014a, 2014b; Rakoff-Nahoum, Coyne and Comstock 2014; Turroni *et al.* 2015).

A prerequisite to enter such intricate nutrient networks is the specialization towards a subset of the available substrates, as is often apparent in the genomes of human gut symbionts (Xu *et al.* 2003; Sela *et al.* 2008; Martens *et al.* 2011). *Lactobacillus reuteri* is a host-specific gut symbiont

to different vertebrate species (Oh *et al.* 2010; Frese *et al.* 2011; Walter, Britton and Roos 2011; Duar *et al.* 2017) (Chapter 2) and one of the few lactobacilli regarded as autochthonous to the human gut (Reuter 2001). Comparisons between the genomes of human and rodent isolates revealed that strains have adapted to the gut conditions of each corresponding host (Frese *et al.* 2011). In rodents, *L reuteri* colonizes by producing host-specific biofilms on the keratinized epithelium of the forestomach. Here it obtains simple carbohydrates and growth factors from the host's diet. (Frese *et al.* 2013). Analogous non-secretory regions, have not been described in the human gastrointestinal tract where *L. reuteri* is likely to be restricted to a planktonic lifestyle (Walter, Britton and Roos 2011). This lifestyle poses important challenges for nutrient acquisition. *L reuteri*, like other lactobacilli, relies on the availability of fermentable sugars for growth but such sugars rarely reach the colon. Therefore, how *L. reuteri* satisfies its growth requirements in the human colon is currently unknown. One interesting finding from genome comparative analyses was that genes involved adhesion and biofilms formation, presumably superfluous to a lifestyle in the human colon, have been largely deleted from the genomes of human strains. However, a 58-gene cluster encoding several biosynthetic enzymes has been conserved in human isolates and mostly absent from the genomes of animal-associated strains (Frese *et al.* 2011).

Encoded in the *pdu-cbi-cob-hem* cluster (*pdu*-cluster, for short) are enzymes involved in the biosynthesis of cobalamin and the broad spectrum antibiotic reuterin (Morita *et al.* 2008; Sriramulu *et al.* 2008). Also in this cluster is the is the glycerol/diol dehydratase PduCDE involved in the utilization of glycerol and 1,2 propanediol (1,2 PD). Metabolism of glycerol improves the competitiveness of *L. reuteri* in sourdough and addition of 1,2 PD increases cell yield of *L. reuteri* growing in galactooligosaccharides (Rattanaprasert *et al.* 2014). In the human intestine, deoxy hexoses, namely rhamnose and fucose, are readily available as part plant-derived dietary fibers and host mucins, respectively. *L reuteri* cannot utilize these sugars directly (Rattanaprasert, 2014). However, a number of intestinal bacteria metabolize fucose and rhamnose into 1,2 PD (Saxena *et al.* 2010; Reichardt *et al.* 2014). Hence, the ability of *L. reuteri* to metabolize 1,2PD

has been hypothesized to be relevant to its ecological performance in the human gut (Walter, Britton and Roos 2011). The primary goal of this project was to determine the role of the *pdu*-cluster in the context of metabolic cross-feeding of 1,2 PD. For this purpose we selected strains from two different 1,2 PD-producing species that are commonly found in the human gut (*Escherichia coli* and *Bifidobacterium breve*) and determined the ability of *L. reuteri* to benefit from 1,2 PD provided via cross-feeding through the fermentation of rhamnose and fucose by other species.

4.2 Materials and Methods

4.2.1 Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. *L. reuteri* strains were grown in MRS medium (Difco) under anaerobic conditions (5% CO2, 5% H2, and 90% N). *B. breve* strains were grown anerobically in Reinforced Clostridial Medium broth (Oxoid Ltd). *E. coli* were grown in Luria-Bertani (LB) broth with agitation. All cultivations were at 37 °C.

Table 4.1 Bacterial strains used in this study

Strain (other names)	Origen	Relevant characteristics	Reference or Source
Bifidobacterium breve UCC2003	Stool of a nursing infant	L-fucose utilizer, 1,2 PD producer	(Mazé *et al.* 2007)
Escherichia coli MG1655	Derived lab strain	L-rhamnose utilizer, 1,2 PD producer	The Coli Genetic Stock Center (CGSC)
Lactobacillus reuteri ATCC PTA 6475 (MM4-1A)	Breast milk	Wild type, 1,2 PD utilizer	BioGaia AB
Lactobacillus reuteri Δ*pduCDE*	Derivative of ATCC PTA 6475	Deletion mutant, glycerol/diol dehydratase PduCDE	This study

4.2.2 Generation of the *L. reuteri* Δ*pduCDE* mutant

A suicide shuttle vector for *pduCDE* deletion (pVPL3478) was constructed by Ligase Cycling Reaction (LCR) as described in de Kok *et al.* (2014). Up-stream flanking (oVPL1335 and oVPL1336, 847bp) and down-stream flanking region (oVPL1337 and oVPL1338, 945bp) from *L. reuteri* ATCC PTA 6475 *pduCDE* locus were amplified by PCR. Backbone of suicide shuttle vector pVPL3002 (3.7kb pORI19_Em^R derivative with vancomycin counter-selection marker) was amplified with oVPL187-188. Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific) by PCR. Amplicons were purified by using Gen-jet PCR-purification kit (Thermo Fisher Scienctific) and vector backbone was subsequently treated with DpnI (Fast Digest, Thermo Fisher Scientific) followed by PCR clean-up. 4 nM of each PCR amplicons were mixed and phosphorylated by using poly nucleotides kinase (Thermo Fisher Scientific) followed by EtOH precipitation. LCR was conducted in PCR machine as described in a previous study (Kok *et al.* 2014) and three bridging oligos (oVPL1339, 1340, and 1341) were used for LCR. LCR ligates were pellet-paint® precipitated followed by *E. coli* EC1000 transformation. 5 µg total plasmid DNA (pVPL3478) was used to transform *L. reuteri* ATCC PTA 6475. *L. reuteri* electro-competent cell preparation and electroporation were performed following protocols described in Oh and Van Pijkeren (2014). Single crossover was screened by PCR from colonies on MRS containing 5 µg/ml erythromycin and subsequently *ΔpduCDE* mutant colonies derived from two passages in plain MRS broth were screened on a MRS plate containing 500 µg/ml vancomycin followed by colony PCR (oVPL1342 and oVPL1344) and sequencing analysis (Table S1).

4.2.3 Basal media for fermentations

The basal MRS (bMRS) was devised based on a recipe described elsewhere (Stolz *et al.* 1995). The bMRS media consisted of (per liter): 5g of peptone, 2.5g of beef extract, 2.5g of yeast extract, 1.5g of ammonium chloride, 1.6g of monopotassium phosphate, 2g of dipotassium phosphate, 0.025 g of magnesium sulfate, 0.0175g of mangase sulfate, 0.25g of L-cysteine

hydrochloride and 0.5 ml of tween 80. Basal media was autoclaved for 15 min at 120°C and the pH adjusted to 6.6.

4.2.4 Screening of 1.2 PD utilization by *L reuteri.*

Utilization of 1,2 PD and the metabolites produced during co-fermentation with glucose were determined for the wild-type *L. reuteri* ATCC PTA 6475 and a *pduCDE* mutant strain (Table 1). Cells from overnight cultures were harvested by centrifugation and washed in sterile PBS (pH 7.0) as previously described (Rattanaprasert *et al.* 2014). Washed cells were suspended in bMRS and inoculated at 1 % into 25 ml of bMRS media containing 20 mM of 1,2 PD alone, 50 mM of glucose alone, or a combination of both. Cell growth was monitored by optical density at 600 nm (OD_{600}). All experiments were performed in triplicate at 37°C under anoxic conditions.

4.2.5 *In vitro* fermentations for 1,2 PD production

Carbohydrates were added separately to the bMRS from filter-sterilized stock solutions as summarized in Table 2. L- rhamnose was added to a final concentration of 40 mM and L-fucose was added to reach 20 mM. Media were left overnight to reduce in the anaerobic chamber. The following morning 200 ml of bMRS containing 40 mM L-rhamnose was inoculated at 1% with *E. coli* grown overnight in LB. *B. breve* grown overnight in MRS (Difco) was added at 1% inoculum to 200 ml of bMRS media containing 20 mM of fucose. Fermentations were conducted for 24 h at 37 °C under anaerobic conditions. Bacterial cells were then removed by centrifugation (5 000 × *g* for 5 minutes). Both media were resuplemented (using solid ingredients) to regain the nutrient and salts concentration of the bMRS, filtered sterilized (0.2 µm), and stored at 4 °C. Cross-feeding assays were conducted within 2 days.

4.2.6 Cross-feeding assays

Growth of *L. reuteri* in media previously fermented by *E. coli* was tested with and without the addition of glucose (25 mM) as summarized in Table 4.2. Because growth of *B. breve* was not achieved in bMRS media, the effect of fucose on the growth of *L. reuteri* was determined in bMRS media containing fucose (20 Mm) in the presence and absence of glucose (Table 4.2). *L. reuteri* inoculums for cross-feeding assays were prepared as described previously (see Methods sections “Screening for 1,2 PD utilization by *L. reuteri*”). Cell growth was monitored for 12 hours. OD_{600} readings and 1 ml samples for HPLC were collected every 3 hours. All experiments were performed in triplicate at 37°C under anoxic conditions.

4.2.7 Analytical methods

The amount of 1,2 PD produced from L-frucose and L-rhamnose and present in the resuplemented media, as well as the production of lactate, acetate, ethanol, propanol and propionate and residual amounts of 1,2PD after fermentations were determined by HPLC (Lin and Gänzle 2014). During the cross-feeding growth assays 1 ml samples were taken for metabolites analysis at 0, 6, and 12 hours.

4.2.8 Statistical analysis

Significant differences in OD_{600} values were determined by Student’s t-tests calculated using GraphPad Prism version 6.07

Table 4.2 List of media used for cross-feeding experiments

Fermenting organism	Before fermentation		Reconditioned for cross-feeding			Purpose
	Deoxy hexose	Approx. 1,2 PD produced [a]	Added glucose	Added deoxy hexose	Abbreviation[b]	
E. coli						
	none	not detected	25 mM	none	Eco + (Glc)	Determine the growth rate of *L. reuteri* in a resuplemented media containing glucose
	none	not detected	none	Rha(40mM)	Eco + (Rha)	Determine if *L. reuteri* can use rhamnose as a growth substrate
	none	not detected	25 mM	Rha(40mM)	Eco+(Rha+Glc)	Determine the effect of rhamnose in co-fermentation glucose on the growth rate of *L. reuteri*
	Rha 40mM	15mM	none	none	Eco_1,2PD	Determine if *L. reuteri* can use 1,2 (produced from Rha) as a growth substrate
	Rha 40mM	15mM	25 mM	none	Eco_1,2PD+(Glc)	Determine the effect of 1,2 PD (produced from Rha) in co-fermentation with glucose, in the growth rate of *L. reuteri*
B. breve						
	Fuc 20mM	15mM	25 mM	none	Bre_1,2PD+(Glc)	Determine effect of 1,2 PD (produced from Fuc) in co-fermentation glucose
Other media						
	none	not detected	none	Fuc(20mM)	bMRS+Fuc	Determine if *L. reuteri* can use fucose as a growth substrate
			25 mM	Fuc(20mM)	bMRS+Fuc+Glc	Determine the effect of fucose in co-fermentation with glucose on the growth rate of *L. reuteri*

[a]as determined by HPLC.
[b]sugars added during the reconditioning step are shown in brackets
Eco: *E. coli*; Bre: *B. breve*
Rha: rhamnose, Fuc: fucose, Glc: glucose

4.3 Results

4.3.1 Effect of 1,2 PD on growth of *L. reuteri*

Growth of *L. reuteri* ATCC PTA 6475 wild type and a *pduCDE* mutant in bMRS containing glucose was determined by OD and compared to growth in this same media supplemented with 1,2 PD. Growth patterns, utilization of 1,2 PD and its effects on glucose metabolism were determined under anaerobic conditions and monitored for 12 hours (Fig 1 a-c). When grown in bMRS with glucose (bMRS+Glc), *L. reuteri* wild type and the *pduCDE* mutant reached comparable ODs (Fig1a). However, when 1,2 PD was added to this media (bMRS+glc+1,2PD) the wild type strain grew faster and reached a higher OD, whereas no change in growth was observed for the *pduCDE* mutant (Fig 1b). As shown in Fig. 1c, addition of only 1,2 PD to bMRS media resulted in no growth for either strain, confirming that *L. reuteri* are unable to utilize 1,2 PD as a sole carbon source.

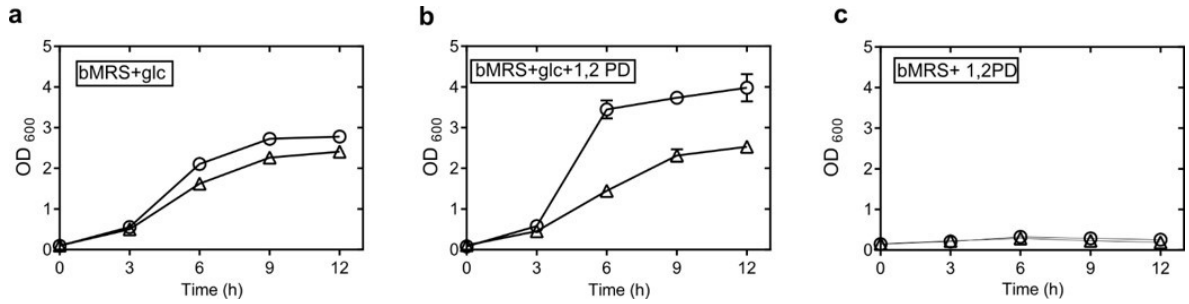

Figure 4.1 Growth characteristics of *L. reuteri* ATCC PTA 6475 and Δ*pduCDE*

Growth of *L. reuteri* ATCC PTA 6475 and ATCC PTA 6475 *Δ pduCDE* was monitored by OD_{600} for 12 hours in bMRS containing (a) glucose (50mM),(b) glucose (50mM) and 1,2 PD (20mM) (c) 1,2 PD (20mM). Results presented are mean values obtained from three separate experiments. Error bars represent standard deviations.

At the end of the fermentation, the concentration of 1,2 PD, propanol and propionate was measured in the culture supernatants by HPLC. As shown in Table 3, almost all the 1,2 PD added to the media was metabolized into propanol and propionate by the wild type strain. With propanol being produced more abundantly than propionate. On the other hand, after 12 h of fermentation with the *pduCDE* mutant, the residual amount of 1,2 PD was only 2mM less than the amount initially added to the media. Production of propanol or propionate was not detected in the culture supernatant of the *pduCDE* mutant strain.

Lactate, acetate and ethanol were also measured in the culture supernatants to determine the effects of 1,2 PD in the metabolism of glucose (Table 3). When 1,2 PD was added to the media, production of ethanol decreased resulting in a concomitant increase of acetate, while production of lactate remained constant. The metabolic end products of glucose fermentation of the *pduCDE* mutant strain were similar to those of the wild type and remained at the same molar ratio independent of the addition of 1,2 PD. Overall, these results indicate that simultaneous fermentation of glucose and 1,2 PD increases the conversion of glucose into acetate, resulting in approximate equimolar amounts of acetate and ethanol, and these effects are abrogated with the inactivation of the glycerol/diol dehydratase *pduCDE*

4.3.2 Production of 1,2 PD from deoxy hexoses

We then sought to determine whether 1,2 PD excreted from a “producer” species could be utilized by *L. reuteri.* For the selection of producer strains we established four criteria (i) the organism must be commonly found in the human gastrointestinal tract (GIT) (ii) be nonpathogenic, (iii) able to produce 1,2 PD from deoxy hexoses commonly present in the GIT under anaerobic conditions, and (iv) be able to grow on bMRS media. This last criteria would come to importance in future co-culture experiments of the 1,2 PD-producer and *L. reuteri.* Based on the above criteria we selected *E. coli* and *B. breve* as the 1,2 PD-producer organisms. Strains of *E coli* can utilize L-rhamnose, which is available in the gut as it is present in food-derived pectic and hemicellulosic

polysaccharides and, under anaerobic conditions, excrete 1,2 PD into the medium (Baldomà and Aguilar 1988). Strains of *B. breve* can utilize L-fucose, which is a component of host-derived glycans, and although production of 1,2 PD from L-fucose had to our knowledge not been demonstrated experimentally, most *B. breve* strains possess the genetic machinery to produce 1,2 PD (Egan *et al.* 2014a; Bunesova, Lacroix and Schwab 2016). For the production of 1,2 PD, *E. coli* and *B. breve* were grown anaerobically in bMRS containing different amounts of L-rhamnose or L-fucose, respectively. The highest yield of 1,2 PD was determined to come from fermentation of 40mM of L-rhamnose and 20mM of L-fucose.

Table 4.3 Effect of addition of 1,2 PD on the end products of heterolactic fermentation of glucose by *L. reuteri*

Strain	Media	Metabolites produced in the presence/absence of 20mM of 1,2 PD			Metabolites produced from fermentation of 55 mM glucose			Approx. molar ratio E:A
		1,2 PD consumed	Propanol	Propionate	Lactate	Ethanol (E)	Acetate (A)	
Wild-type								
	bMRS	-	-	-	33 ± 3.8	18.1 ± 4.8	1.0 ± 0.2	18:1
	bMRS+1,2PD	-16.5+0.2	14.6 ± 2.9	2.7 ± 0.1	29.9 ± 1.2	9.7 ± 1.9	7.4 ± 0.2	1:1
Δ*pduCDE*								
	bMRS	-	-	-	33.7 ± 0.5	20.3 ± 0.4	0.94± 0.00	20:1
	bMRS+1,2PD	-2.1 ± 1.3	n.d	n.d	26.4 ± 1.2	13.8 + 1.8	0.80 ± 0.08	17:1

n.d, not detected

4.3.3 Cross-feeding of *E. coli*-produced 1,2 PD

The next step was to determine whether *L. reuteri* could grow on a filter sterilized reconditioned medium derived from growth of a 1,2 PD-producing species. As shown in Fig 4.2a, both the *L. reuteri* ATCC 6475 and the *pduCDE* mutant are able to grow on an *E. coli* reconditioned media containing glucose [Eco+(Glc)] and reached comparable ODs. We also determined the effects of rhamnose on the growth of *L. reuteri*. As shown in Fig 4.2b, *L reuteri* strains were unable to grow when the reconditioned media contained only rhamnose [Eco+(Rha)]. Furthermore, addition of rhamnose had no effect in the growth of *L. reuteri* in glucose [Eco+(Rha+Glc)] (Fig 4.2c) as the highest OD reached was equivalent in the absence (wild-type 2.6 ± 0.1, Δ*pduCDE* 2.5 ± 0.1) or presence (wild-type 2.4 ± 0.1, Δ*pduCDE* 2.3 ± 0.1) of rhamnose. Finally, we confirmed that neither the wild type or the mutant strain were able to grow on reconstituted media containing 1,2 PD produced from the fermentation of L-rhamnose as the sole substrate (Fig 4.2d).

Having confirmed that *L. reuteri* could grow on an *E. coli*-reconditioned media and that L-rhamnose had no effect on growth, we continued with the cross-feeding experiments. The growth of *L reuteri* wild-type and the *pduCDE* mutant were monitored for 12 hours in a reconditioned media containing *E.coli*-produced 1,2 PD and 25mM of glucose [Eco_1,2PD +(Glc)]. As shown in Fig. 3, both mutant and wild type strains grew to the same OD during the first 6 hours. However at the 9 hour time point the wild-type strain reached a significantly higher (p<0.05) OD than the Δ*pduCDE* strain. This difference in OD was also observed at the 12 hour time point (Fig 3a).

Analysis of the 1,2 PD, propanol and propionate in culture supernatants revealed that in co-fermentation with glucose, the wild-type strain metabolized 1,2 PD into propanol and propionate, whereas the *pduCDE* mutant was unable to metabolize 1,2 PD (Fig.3 b-d) Overall these analyses confirm that *L. reuteri* can cross-feed from 1,2 PD produced by the fermentation of L-rhamnose by *E. coli.*

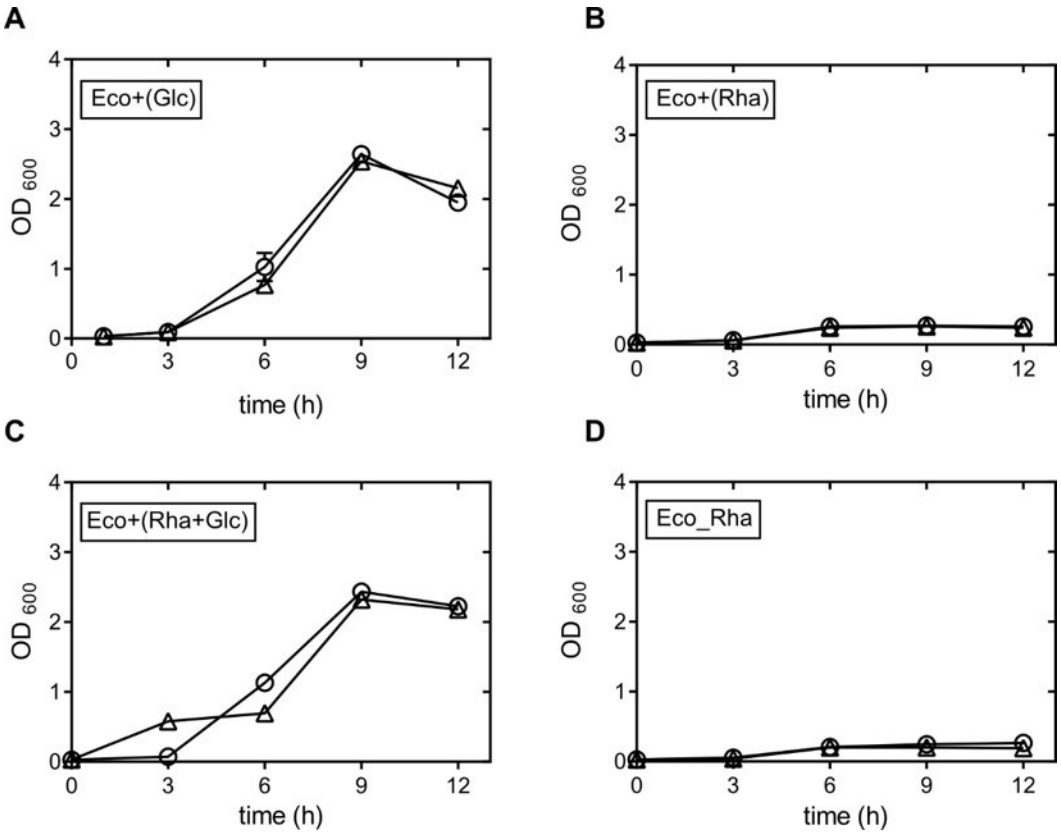

Figure 4.2 Growth characteristics of *L. reuteri* ATCC PTA 6475 (circles) and PTA 6475 Δ*pduCDE* (triangles) in a reconditioned *E. coli* spent medium

Growth of *L. reuteri* ATCC PTA 6475 and the PTA 6475 the *pduCDE* mutant was monitored by OD for 12 hours in spent medium in which *E. coli* has been previously grown and reconditioned to contain (a) glucose (25mM), (b) rhamnose (40mM) (c) rhamnose and glucose (d) only 1,2 PD produced from the fermentation of L-rhamnose. Results presented are mean values obtained from three separate experiments. Error bars represent standard deviations.

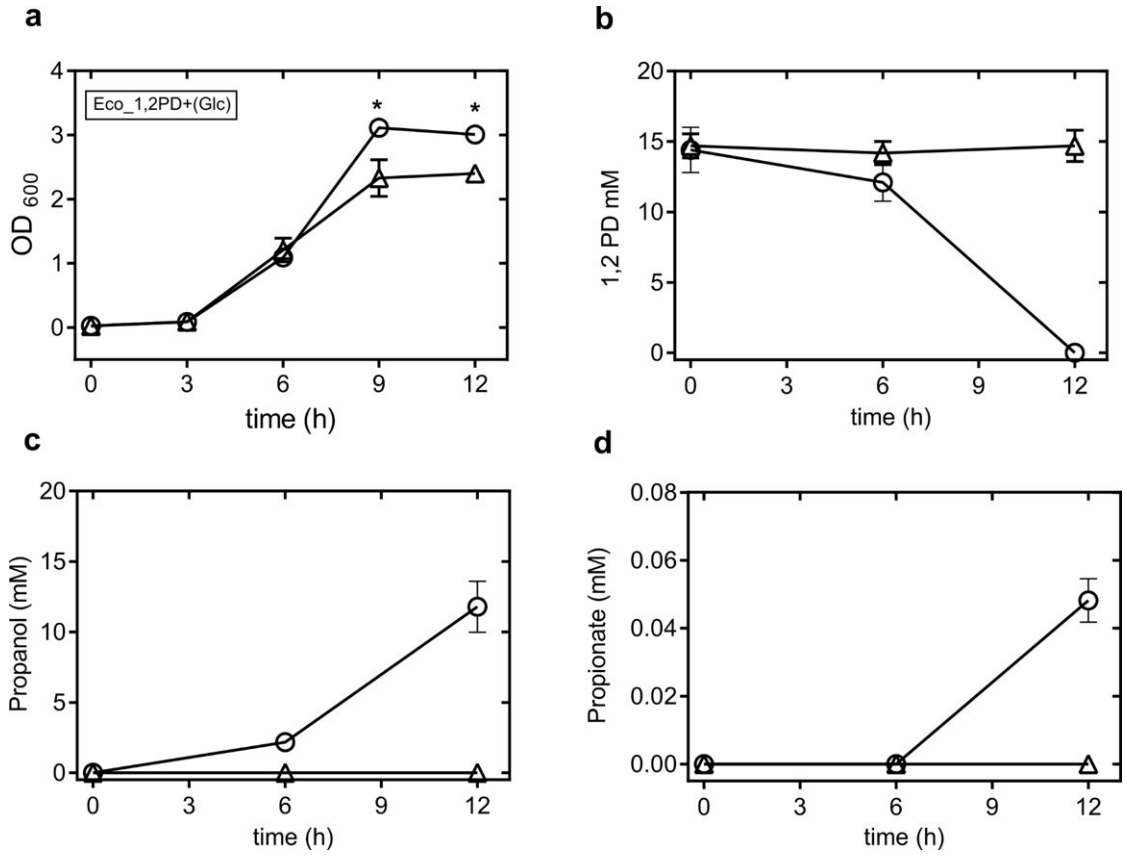

Figure 4.3 Growth and metabolites produced by *L. reuteri* ATCC PTA 6475 (circles) and PTA 6475 Δ*pduCDE* (triangles) growing in a reconditioned *E. coli* spent medium

(a)Growth as determined by OD_{600} (b) utilization of 1,2 PD, and production of (c) propanol and (d) propionate. Results presented are mean values obtained from three separate experiments. Error bars represent standard deviations. Significant differences (p<0.05) in OD values are denoted with an asterisk

4.3.4 Cross-feeding of *B. breve*-produced 1,2 PD

First we sought to determine if addition of L-fucose had any effects on the growth of *L. reuteri.* As shown in Fig. 4a, *L. reuteri* strains were unable to grow on bMRS supplemented with L-fucose alone (bMRS+Fuc). Additionally, both wildtype and mutant grew to comparable ODs in bMRS containing both glucose and L-fucose (bMRS+Glc+Fuc) (Fig 4b). Furthermore, neither *L. reuteri* strain was able to grow in in a reconditioned *B. breve* media containing only 1,2 PD produced form L-fucose (Fig. 4c). Based on these findings we concluded that L-fucose had no effects in the growth of *L reuteri a*nd confirmed that strains cannot use 1,2 PD as carbon source. We then continued with the cross-feeding experiments.

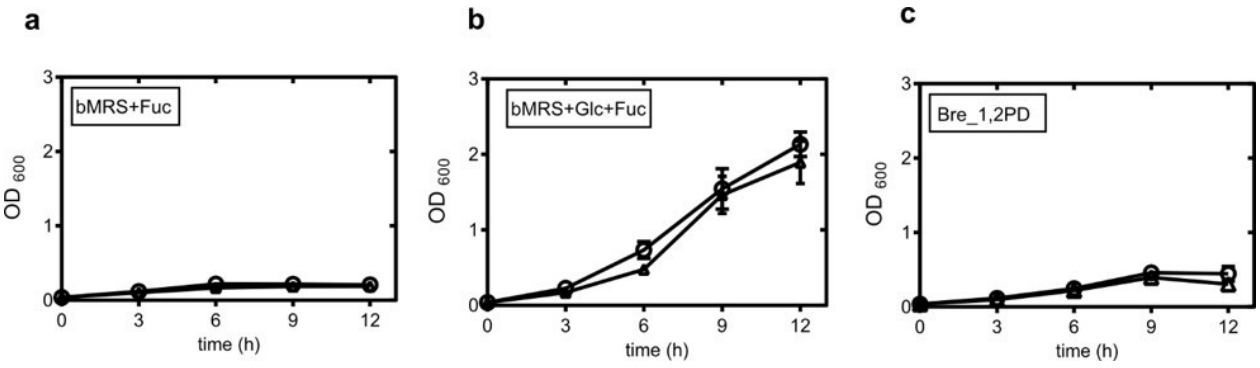

Figure 4.4 Growth characteristics of *L. reuteri* ATCC PTA 6475 (circles) and PTA 6475 Δ*pduCDE* (triangles) bMRS and in a reconditioned *B. breve* spent medium

Growth in was monitored by OD_{600} for 12 hours in (a) bMRS containing fucose (b)bMRS containing glucose and fucose, and in a (c) a reconditioned *B. breve* medium containing 1,2 PD from fermentation of L-fucose. Results presented are mean values obtained from three separate experiments. Error bars represent standard deviations.

As shown in Fig. 5 different growth profiles were observed when glucose was added to the reconditioned media containing 1,2 PD produced from the fermentation of L-fucose [Bre_1,2PD+(Glc)]. During the first 6 hours of growth on this media, both mutant and the wild-type strains reached equivalent ODs (Fig 5 a). However, after 9 hours the mutant appeared to have reached stationary while the wild-type strain continued growing, reaching a significantly higher (p<0.05) ODs at the 9 hour time point and the 12 hour time point (Fig 5 a). Quantitative analysis of 1,2 PD, propanol and propionate showed that 1,2 PD was metabolized into propanol and

propionate by the wild type strain (Fig 5. b-d). Conversely, the *pduCDE* mutant was not capable of utilizing 1,2 PD as evidenced by the residual amount of 1,2 PD in the supernatant (Fig 5. b).

Taken together these findings show that *L. reuteri* can cross-feed from 1,2 PD produced from the fermentation from deoxy hexoses by two common GIT inhabitants, *E. coli* and *B.breve.* In co-fermentation with glucose, disproportionation of 1,2 PD into propanol and propionate increases the growth of *L reuteri* was determined by OD. Inactivation of the glycerol/dehydratase *pduCDE* abrogates such effects.

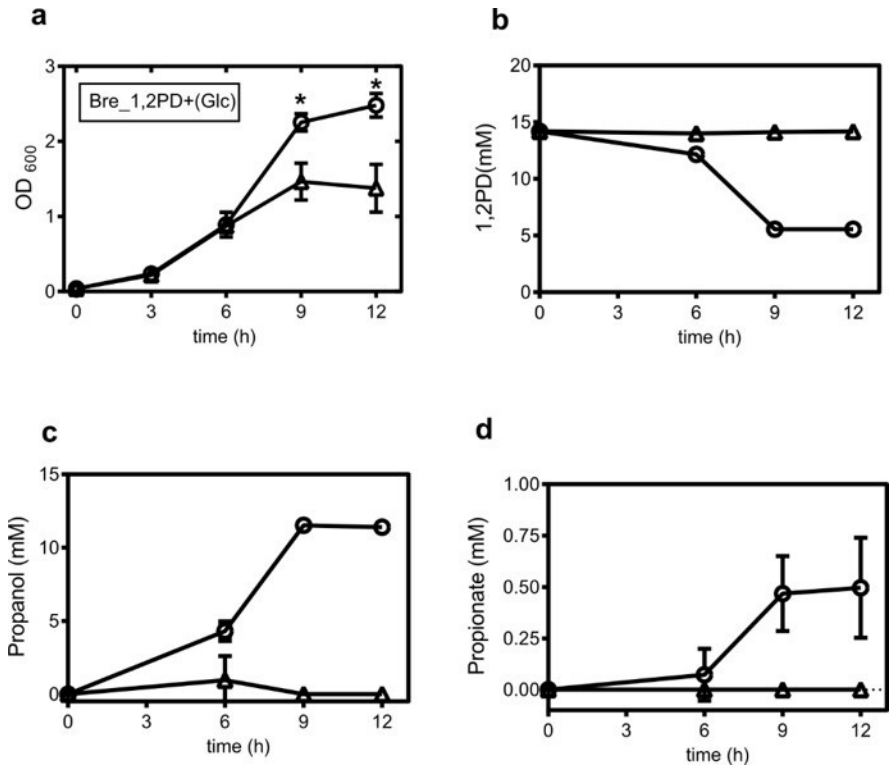

Figure 4.5 Growth and metabolites produce by *L. reuteri* ATCC PTA 6475 (circles) and PTA 6475 Δ*pduCDE* (triangles) growing in a reconditioned B. breve spent medium

(a)Growth as determined by OD_{600} (b) utilization of 1,2 PD, and production of (c) propanol and (d) propionate. Results presented are mean values obtained from three separate experiments. Error bars represent standard deviations. Significant differences (p<0.05) in OD values are denoted with an asterisk

4.4 Discussion

Substrate breakdown by intestinal bacteria involves the combined metabolic action of primary fermenters and syntrophs that consume generated byproducts (Fischbach and Sonnenburg 2011; Koropatkin, Cameron and Martens 2012). Here we demonstrate that trophic interaction of metabolites can occur between human isolates of *L. reuteri* and strains from two common intestinal species, *E. coli* and *B. breve*. We further demonstrate that this interaction is facilitated by the *pdu* cluster, and more specifically, the propanediol dehydratase PduCDE, which allows *L. reuteri* to cross feed on fermentation end products (1,2 PD) of rhamnose and fucose.

One major determinant to the success of a bacterium in a particular niche depends largely on its ability to obtain resources to generate energy. *L. reuteri,* like other heterofermentative lactobacilli, generates ATP from carbohydrates via the phosphoketolase pathway (PKP) (Arskold et al. 2008). In the absence of electron acceptors to regenerate reduced cofactors, the PKP is energetically inefficient (Gänzle 2015). In the mouse forestomach, *L. reuteri* obtains fermentable sugars and electron acceptors (e.g. fructose) from the digesta (Tannok 2012). These compounds are much harder to come across in the distal portions of the human intestine, as they become absorbed early in the digestive process. The main sources of carbohydrate in the human colon are dietary polysaccharides and glycosylated host secretions (Koropatkin, Cameron and Martens 2012). However, *L reuteri* is poorly equipped to degrade most of these carbohydrates (Gänzle and Follador 2012). The success of *L. reuteri* in the human gut might therefore depend on its ability to thrive on the remnants of primary fermenters, such as bifidobacteria, which are better equipped to utilize resources available in the distal intestinal milieu (Schell et al. 2002; Pokusaeva, Fitzgerald and van Sinderen 2011; Sela et al. 2012).

Rhamnose forms part of the structural polysaccharides of the primary cell wall of plants (Yapo 2011). Fucose is abundant in human secretions as part of human milk oligosaccharides, mucins and other glycoconjugates in the intestinal epithelium (Becker and Lowe 2003; Zivkovic et al. 2011). Both of these deoxyhexoses reach the colon and are fermented into 1,2 PD by a number

of intestinal bacteria, making this compound readily available in the human gut (Saxena et al. 2010; Reichardt et al. 2014). Our results demonstrate that *L. reuteri* can obtain a growth advantage by crossfeeding from 1,2 PD produced by *B. breve* and *E. coli* from the fermentation of fucose and rhamnose, respectively. These results are in accordance with previous findings that show that in co-fermentation with glucose, 1,2 PD (and glycerol) increase the metabolic rate or *L. reuteri* resulting in higher cell yield (Talarico et al. 1990; Lin and Gänzle 2014; Rattanaprasert et al. 2014). By generating a knockout mutant we confirm that disproportionation of 1,2 PD into propanol and propionate is facilitated by the propanediol dehydratase PduCDE which is encoded in the human specific *pdu*-cluster (Morita et al. 2008; Frese et al. 2011). Analysis of the culture supernatants revealed that propanol is produced more abundantly than propionate (Table 3, Fig 3 and 5). This confirms that 1,2 PD serves as a hydrogen acceptor to recover NAD+, thus allowing *L. reuteri* to spare the acetyl-phosphate to generate an extra ATP via production of acetate.(Table 3, Fig 6).

In the highly competitive ecosystem of the gastrointestinal tract, generating energy more efficiently can be key to the persistence of *L. reuteri* and might explain why human isolates have conserved the *pdu*-cluster (Frese et al. 2011). In this sense, it is important to point out that although *L. reuteri* has been regarded as autochthonous to the human gut (Reuter 2001), its prevalence appears to have reduced substantially in individuals of industrialized societies (Walter, Britton and Roos 2011). Interestingly, a recent study found *L. reuteri* to be a dominant member of the fecal microbiome of natives of agriculturalist tribes consuming high fiber diets (Martínez et al. 2015). Research in mice has shown that insufficient supply of microbiota-accessible carbohydrates can irreversibly deplete species from the gut in just a few generations (Sonnenburg et al. 2016). The average modern diet is alarmingly low in fiber (Deehan and Walter 2016). A low intake of fiber would not only have consequences for primary fermenters reliant of these substrates for growth, but would also inevitably result in a decreased production of end products. It is therefore possible that modern diets do not provide an adequate supply of fiber-derived

deoxyhexoses (i.e. rhamnose), resulting in a decreased production of 1,2 PD in the gut. This could mean that the niche conditions in which *L. reuteri* evolved with humans are no longer existent and would explain why human strains do not persist in the human, as shown in Chapter 2.

Taken together, findings generated in this study serve as proof-of-concept that a human-strain specific function might be relevant to the persistence to *L. reuteri* in the colon and provide novel insight into the molecular mechanisms that could underlie the association of *L. reuteri* with humans. Thus far, the relevance of *pdu*-cluster in human isolates of L. reuteri has been studied in the context of reuterin production and glycerol utilization (Lin and Gänzle 2014; Spinler et al. 2014). This is to our knowledge, the first demonstration of the relevance of the pdu-cluster for cross-feeding of 1,2D.

In vitro fermentations have proven to be excellent tools to study the metabolic underpinnings of cross-feeding amongst intestinal bacteria (Falony et al. 2006; Rakoff-Nahoum, Coyne and Comstock 2014; Rivière et al. 2015; Turroni et al. 2015; Moens, Weckx and De Vuyst 2016; Schwab et al. 2017). Nevertheless, these experiments are limited in that they do not replicate the intricacy of the intestinal ecosystem. Seminal findings regarding trophic interactions in the gut have been obtained using simplified in vivo models of cocolonization and in humanized mice (Samuel and Gordon 2006; Mahowald et al. 2009). Future studies using similar approaches are thus warranted to determine of the relative importance of cross-feeding of 1,2 PD for *L. reuteri* in gut conditions.

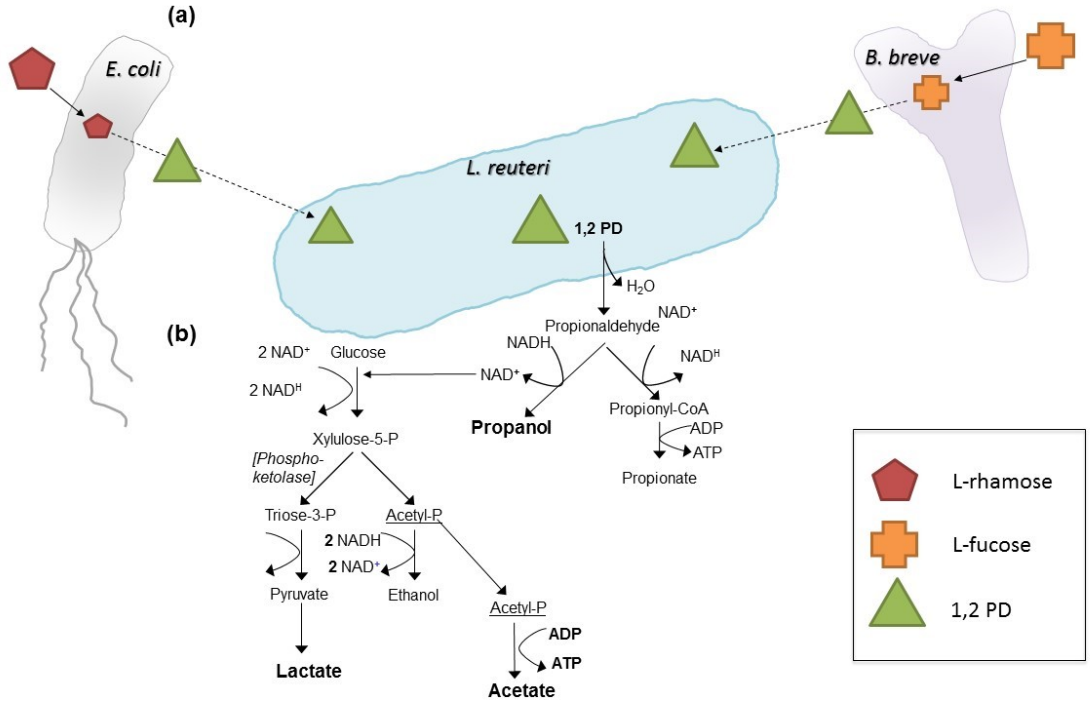

Figure 4.6 Illustrative and schematic representation 1,2 PD cross-feeding and glucose metabolism in the presence of 1,2 PD

(a) Illustrative representation of metabolic cross-feeding between 1,2 PD producers (*E. coli* and *B breve*) and *L. reuteri*. Dotted lines indicate cross-feeding of 1,2 PD. (b) General scheme of the heterofermentative metabolism of hexoses via de PKS pathway and 1,2 PD utilization by *L. reuteri*. The presence of 1,2 PD relieves the need for to utilize acetyl phosphate (acetyl-P) as a hydrogen acceptor to generate NAD^+, thereby sparing this metabolite for ATP production. Major metabolic end products are printed in bold. Metabolic pathways drawn according to (Gänzle 2015)

4.5 Supplementary material

Table S.4.1 Oligonucleotides and plasmids used to generate the *pduCDE* mutant

Oligonucleotides	Sequence	Description
oVPL187	TACCGAGCTCGAATTCACTGG	pVPL3002 Backbone, Rev
oVPL188	ATCCTCTAGAGTCGACCTGC	pVPL3002 Backbone, Fwd
oVPL1335	GGTTCTTGAAATGTATGATGCA	5' u/s flanking region of pduCDE, Fwd
oVPL1336	ACAACAGCCGAACGATTTCC	5' u/s flanking region of pduCDE, Rev
oVPL1337	TGGTGCTAGCGAAATTGGAG	3' d/s flanking region of pduCDE, Fwd
oVPL1338	TACGATCTTGCCATTTTCAAC	3' d/s flanking region of pduCDE, Rev
oVPL1339	AGTGTAAAAGTTGAAAATGGCAAGATCGTAGG TTCTTGAAATGTATGATGCATTGCGTCC	Bridging oligo for LCR
oVPL1340	AAACGACGGCCAGTGAATTCGAGCTCGGTATG GTGCTAGCGAAATTGGAGATACCATTGG	Bridging oligo for LCR
oVPL1341	AGCGCTTATTGGAAATCGTTCGGCTGTTGTATC CTCTAGAGTCGACCTGCAGGCATGCAA	Bridging oligo for LCR
oVPL1342	AGTTGATGCCGGAGTACAAG	u/s SCO screening, Fwd
oVPL1343	TGGCGTGGCTTCATTGATTC	u/s SCO screening, Rev
oVPL1344	ACATTGGTTCCAGACTCACCAG	d/s SCO screening, Fwd
oVPL1345	ATGGCTGGACGTGAAGTAGG	d/s SCO screening, Rev (sequencing)
oVPL1346	TGAAGCCACGCCAGTAATTG	(sequencing)
oVPL1347	TGCAACGAAACCTTCTTCTGG	(sequencing)
Plasmids		
pVPL3002		pORI19_Em^R derivative with vancomycin counter-selection marker
pVPL3478		pVPL3002 ::*ΔpduCDE cassette*

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5. Chapter five: Identification and characterization of intestinal lactobacilli strains capable of degrading immunotoxic peptides present in gluten.

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5.1 Introduction

Celiac disease is an increasingly diagnosed enteropathy caused by an uncontrolled immune response to wheat gluten and homologous proteins in barley and rye (Sollid 2002; Ludvigsson *et al.* 2013). The key T-cell stimulatory peptides responsible for celiac disease escape breakdown by gastrointestinal (GI) proteases and reach the small intestine where they cross the epithelial barrier to the lamina propria. The peptides become deaminated by the enzyme tissue transglutaminase and activate HLA-DQ2 and HLA-DQ8 restricted populations of CD4+ T cells, leading to inflammation and tissue damage (Green and Cellier 2007). Significant progress has been made in defining the epitopes involved in the pathogenesis of celiac disease (Arentz-Hansen et al. 2000; Shan et al. 2002; Shan et al. 2005; Tye-Din et al. 2010). Despite the absence of a single pathogenic motif, the gluten peptides involved in celiac disease are generally very rich in proline and glutamine residues, a feature that contributes to their resistance against proteolysis in the human gut (Shan *et al.* 2002).

The only available treatment for celiac patients is a strict life-long gluten-free diet. Compliance to a gluten-free diet represents a social and economic burden and the extensive use of gluten in processed foods, cross contamination, and improper labeling, hinders complete avoidance (Di Sabatino and Corazza 2009). Recent studies have improved our understanding of the molecular basis of celiac disease and provided several targets for novel treatments (Sollid and Khosla 2005; Kaukinen, Lindfors and Mäki 2014). One such approach might be the enzymatic detoxification of immunotoxic peptides during GI transit (Bethune and Khosla 2012). To date, enzymes evaluated for this purpose have been derived from plants, fungi, or bacteria that do not originate from GI environments (Piper, Gray and Khosla 2004; Shan *et al.* 2004; Siegel *et al.* 2006; Stepniak *et al.* 2006; Gass *et al.* 2007; Mitea *et al.* 2008). Although several of these enzymes efficiently detoxify gluten epitopes *in vitro*, their activity in the human GI tract remains to be assessed. To our knowledge, very few clinical trials have been conducted (Tack *et al.* 2013) and only one study has been successful (Lähdeaho *et al.* 2014)

The key to a complete removal of gluten toxic peptides in the small intestine might lie in the metabolic arsenal of the bacteria that naturally colonize the GI habitats. The mammalian GI tract harbors a complex, diverse and metabolically active bacterial community (Neish 2009). These microbes are capable of metabolizing complex dietary substrates non-digestible by the human host (Louis *et al.* 2007), and have been exposed to resistant gluten peptides for millennia. They are therefore likely to have evolved strategies to transport, internalize and utilize gluten peptides to satisfy their amino acid requirement (Davila *et al.* 2013). Supporting this hypothesis gluten degrading bacteria from the upper (Zamakhchari *et al.* 2011; Fernandez-Feo *et al.* 2013) and lower (Laparra and Sanz 2010; Caminero *et al.* 2014) human GI tract have been recently discovered. Moreover, imbalances in the composition and metabolism of the gut microbiota of children suffering from celiac disease have been reported, suggesting a potential role of gut bacteria in disease etiology (Tjellström et al. 2005; Nadal et al. 2007; Sanz et al. 2007). These findings clearly warrant further studies on both metabolic and immunologic aspects of gut bacteria in relation to celiac disease. Such bacteria could be used as probiotics with the goal to either remove epitopes before they reach the intestinal mucosa, to promote epithelial healing, or to directly target pathological immune responses (Lindfors et al. 2008; Laparra and Sanz 2010; de Sousa Moraes et al. 2014)

The goal of this study was to identify and characterize bacterial isolates from the proximal GI tract of pigs capable of degrading peptides involved in the etiology of celiac disease. Pigs were used in our study as these animals, in contrast to humans, harbor high numbers of lactic acid bacteria (LAB), especially lactobacilli and streptococci, in their stomach and small intestine (up to 10^9 bacteria/gram content) (Castillo *et al.* 2007). These bacteria are adapted to the conditions in the proximal GI tract and might be good candidates for probiotics aimed at removing gluten epitopes before they reach the epithelium of the small intestine in celiac patients. We successfully identified four strains of lactobacilli that showed degradation of several well characterized gluten immunotoxic peptides (Shan et al. 2002; Tye-Din et al. 2010). We tested the peptide degradation

activity of these strains alone or in combination and we also compared it with strains commonly used as probiotics.

5.2 Materials and Methods

5.2.1 Animals and intestinal sampling

Housing, and management of animals was according to guidelines set forth by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee and described in animal care protocol #07-04-014D.

Weaned pigs (n = 6, initial BW = 4.5 - 7.0 kg; 10 weeks of age) were fed a basal corn-soy diet supplemented with 20% gluten (Vital Wheat Gluten, ADM Milling Co) for at least 16 weeks to stimulate gluten-utilizing bacterial populations in the GI tract. Animals were sacrificed at 26 weeks at the UNL Loeffel Meat Laboratory (a USDA-inspected facility) according to approved procedures. Immediately after sacrifice, 100 ml of proximal small intestinal (SI) contents were collected from each animal. Large debris was removed from the SI contents by low speed centrifugation (200 x *g* for 5 min). Samples were then centrifuged at 6000 x *g* for 20 min and pellets resuspended in 5 ml PBS. Aliquots of 1.5 ml were mixed with 0.5 ml of 60% glycerol and stored at -80 °C.

5.2.2 Enrichment of gluten utilizing bacteria

To enrich for strains capable of metabolizing gluten peptides, a gluten peptide enrichment (GPE) media was devised. This medium was a chemically defined minimal media as described by Elli et al. (2000) with some modifications. The amino acids glutamine, leucine, proline, phenylalanine, and tyrosine were replaced by 5 g/l of the 18-mer GLIAα-2/9 peptide LQLQPFPQPQLPYPQPQL (Arentz-Hansen *et al.* 2000), which is also part of the 33-mer (Shan *et al.* 2002). The GPE media contained glucose 10.0 g/l, yeast extract 0.5 g/l, potassium hydrogen phosphate 3.1 g/l, di-ammonium hydrogen citrate 2.0 g/l, potassium di-hydrogen phosphate 1.5

g/l, sodium chloride 0.02 g/l[1], ascorbic acid g/l, potassium acetate 10.0 g/l, tween 80 1.0 g/l, heptahydrated magnesium sulphate 0.5 g/l, hydrated manganese sulphate 0.02 g/l , cobalt sulphate 0.01 g/l, calcium lactate 1.0 g/l, DL-2-aminobutyric acid 0.1 g/l, L-serine 0.1 g/l, L-threonine 0.1 g/l, L-cysteine 0.1 g/l, L-asparagine 0.1 g/l, L-isoleucine 0.1 g/l, L-methionine 0.1 g/l, L-tryptophan 0.1 g/l, L-valine 0.1 g/l , DL-alanine 0.2 g/l, L-aspartic acid 0.3 g/l, glycine 0.2 g/l, L-histidine-HCL 0.2 g/l, L-lysine-HCL 0.2 g/l, L-arginine 0.2 g/l, guanine 0.1 g/l, thymine 0.1 g/l, cytidine 0.1 g/l, 2'-deoxyadenosine 0.1 g/l and 2'-deoxyuridine 0.1 g/l. $FeSO_4$ was added at the concentration of 0.02 g/l immediately before using the media. The pH was adjusted to 6.9 to resemble small intestine conditions. Frozen stocks were pooled, centrifuged (6000 x *g*, 5 min), and supernatants removed. Pellets were resuspended in 1.0 ml of PBS and used to inoculate the GPE medium at 0.8%. Enrichment cultures were incubated anaerobically at 37 ºC for 24 hours, before a second enrichment step was performed with 1% inoculum under the same conditions. Bacterial population dynamics during the enrichment process with and without the addition of the peptide were characterized by denaturing gradient gel electrophoresis (DGGE) as previously described (Martínez *et al.* 2009).

5.2.3 Culture techniques and classification of isolates

Bacteria were cultured from fresh SI contents and from the 24 hour and 48 hour GPE, by dilution plating on Rogosa (selective for lactobacilli), BEA (enterococci), M17 (non-selective, isolation of streptococci), and BHI (universal) agar. Colonies were picked and grown at least once in liquid media before dilution streaking to assure purity for preparation of stock cultures. Chromosomal DNA from pure cultures was extracted using the DNeasy® Blood and Tissue kit (Qiagen) with modifications according to Oh et al. (2010). Isolates were typed by RAPD-PCR as described by Meroth et al. (2003). Cultures with unique RAPD patterns were taxonomically classified by 16s RNA gene sequencing and database comparisons using the Ribosomal

Database Project SEQ MATCH tool (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and the NCBI nucleotide Blast web tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

5.2.4 Initial screen of isolates for specific proteolytic activities

The chromogenic substrates Suc-Ala-Pro-*p*-NA, Leu-*p*-NA and Pro-*p*-NA (Sigma Chemical Co. St Lois, MO, USA) were used to screen isolates for the production of prolyl endopeptidase (PEP), aminopeptidase type N (PepN), and proline iminopeptidase (PepI), respectively. Proteolytic activities were tested as described previously (Rollán *et al.* 2005), with some modifications**.** Bacterial strains were grown in their respective media and sub-cultured twice. Twelve hours cells were harvested by centrifugation (7000 x *g* for 7 min at 4 °C) and washed twice with 0.05 mol/l tris buffer (pH 7.0). Cells were re-suspended in 1.0 ml of lysis buffer (sucrose 24%, lysozyme 10.0 mg/ml and mutanolysin 50 U/ml) and incubated at 37 °C for 30 min. Cell walls were disrupted using bead beating for 2 min followed by centrifugation (7000 x *g* for 10 min at 4 °C) to obtain cell-free extracts. Protein concentration was determined using the Bradford test and standardized to 2.5 mg/ml. The enzyme reaction mixture contained 360 µl of 0.02 mol/l phosphate buffer (pH 7.0), 150 µl of substrate (0.007 mol l^{-1}), 8 µl of NaN_3 (0.05% final concentration), and 50 µl of cytoplasmic extract. Samples were incubated at 37 °C. Release of the *p*-nitroaniline molecule was measured spectrophotometrically at 410 nm at zero and after four hours.

5.2.5 Immunotoxic peptides

Peptides with a demonstrated importance in the etiology of celiac disease were synthesized by Bio-Synthesis Inc. (Texas, USA). Included in this study were α-gliadin derived peptides 33-mer LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (Shan *et al.* 2002), and 16-mer QLQPFPQPQLPYPQPQ (Tye-Din et al. 2010), and the ω-gliadin/C-hordein derived 17-mer QPQQPFPQPQQPFPWQP (Tye-Din et al. 2010).

5.2.6 Bacterial strains, culture media, and growth conditions

In addition to the bacteria isolated from the pig SI contents and the GPE enrichment during this study, the following commercially available probiotic strains were included: *Lactobacillus plantarum* 299v, *L. casei* ATCC 334, *L. acidophilus* NCFM, *Bifidobacterium animalis* BB-12, *L. reuteri* ATCC 55730, *L. rhamnosus* GG and the probiotic preparation VSL#3 containing, *L plantarum, L. acidophilus, L. casei, L. delbrueckii* spp*. bulgaricus*, *Streptococcus thermophilus, B. breve, B. longum and B. infantis*. Bifidobacteria and lactobacilli strains were cultured anaerobically on MRS and modified MRS media (MRS supplemented with 10.0 g/l maltose and 5.0 g/l fructose), respectively. *Streptococcus thermophilus* was grown on M17 media under aerobic conditions. All incubations were conducted at 37°C.

5.2.7 Peptide degradation

Peptide degradation was determined *in vitro* under conditions reflective of the small intestine. In brief, one colony from a 48 hour agar plate was transferred into broth media and incubated overnight. Fresh media was inoculated with 1% of the overnight culture and incubated for 16 hours. Cells were collected by centrifugation and individual isolates or an even mixture of the strains were re-suspended to an OD_{620} of 2.0 in minimal media (Elli *et al.* 2000) at pH 7.0 containing glucose 10.0 g/l, potassium hydrogen phosphate 3.1 g/l, di-ammonium hydrogen phosphate 2.0 g/l, potassium dihydrogen phosphate 1.5 g/l, sodium chloride 0.02 g/l, ascorbic acid 0.5 g/l, potassium acetate 10.0 g/l, calcium lactate 1.0 g/l, and 1.0 mg/ml of one immunotoxic peptide as the sole amino acid source. Samples were incubated anaerobically with constant shaking (150 rpm) at 37 °C. After 24 hours, bacterial cells were removed by centrifugation (10000 x *g* for 10 min at 4 °C) and trifluoroacetic acid was added to a final concentration of 0.1% (v/v) to stop enzymatic reactions. Control samples without bacterial cells were prepared in parallel and

collected at zero and after 24 hours under the same conditions as test samples. All samples were stored at -20 °C until analyzed.

5.2.8 ELISA tests

The concentration of all three peptides was determined with the RIDASCREEN® (R-Biopharm AG, Darmstadt, Germany) competitive ELISA kit. The peptides 33-mer and 16 mer were also quantified with the GlutenTox® (Biomedal, Seville, Spain) ELISA kit. Samples were analyzed according to the manufacturer's instructions, but quantification was performed using standard curves generated with the respective peptides (Tables S1 – S2). Degradation was calculated as the percent decrease in peptide concentration (µg/ml) in the 24 h test sample relative to the 24 h control sample (without bacterial inoculum).

5.2.9 Mass Spectrometry

Peptide degradation during the GPE enrichment and during the initial screening process was measured by ultrahigh performance liquid chromatography and tandem mass spectrometry in multiple reaction monitoring mode (UHPLC-MS/MS-MRM) at the Proteomics and Metabolomics Core Facility, Department of Biochemistry (University of Nebraska, Lincoln, USA). The UHPLC (Agilent Technologies, Palo Alto, CA) was coupled to a triple quadrupole mass spectrometer (Q-Trap 4000, AB SCIEX) and operated in positive ion ESI-MRM more. Gradient reverse phase chromatography separation of the peptide was carried out at a flow rate of 300 µl min^{-1}. Peptides were eluted on 0.1% formic acid (buffer A) over a 15 min interval using a mixed gradient (0 min = 1%, 7 min = 95%, 10 min = 1%) of buffer B containing 0.1% formic in acetonitrile. Injection volume was 10 µl. Analyst 1.4.2 software was used for instrumentation control and for MRM quantitative analysis based on calibration curves.

Amino acid sequences of the fragments originating from peptide hydrolysis by the four strains selected during this study were determined at the Mass Spectrometry Facility at the National

Jewish Medical and Research Center (Denver, CO, USA) using a quadrupole time of flight mass spectrometer (Agilent Technologies. Palo Alto, CA) equipped with an HPLC-Chip Cube system operated in mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) modes. In brief, aliquots (10 µl) from the peptide test samples and the 24 hour control were vacuum dried at 45 ºC and stored at -20 ºC. Samples were resuspended in 100 µl of 3% acetonitrile + 0.1% formic acid in water. Injection volumes were 0.5 µl and 2.0 µl for the LC-MS and LC-MS/MS analysis, respectively. Peptides were separated with a gradient (0 min = 3%, 0.5 min = 10%, 7 min = 50%, 7.1 min = 80%, 9 min = 80%, 10 min = 3%) of buffer A, containing 0.1% formic acid, and buffer B containing 0.1% formic acid in 90% acetonitrile, at a flow rate of 0.45 µl/min. Mass, abundance and retention time were obtained from the LC-MS analysis using the Agilent Technologies Mass Hunter Qualitative Analysis software, version B.06.00. Fragment sequences were obtained by searching an in-house library using the Spectrum Mill software (Rev A.03.03.038 SR1). Cleavage sites were determined based on fragment sequence and abundance (peak area) (Tables S5 – S7).

5.2.10 Statistical analysis

Initial screening for specialized proteolytic enzymes, ELISA tests and UHPLC-MS/MS-MRM analysis were carried out in duplicate. RIDASCREEN® ELISA data from the selected strains and the commercial probiotics were analyzed in triplicate. All data are shown as the average ± standard deviation. Peptide fragment abundance was calculated as the average peak area from the LC-MS/MS analysis of two independent samples. Correlations between peptide peak area (UHPLC-MS/MS-MRM) and staining intensity of the DGGE fragments were assessed by Pearson's correlation test ($\alpha = 0.05$) using GraphPad Prism version 6.04 (GraphPad Software, San Diego, CA).

5.3 Results

5.3.1 Isolation of intestinal bacteria directly from the proximal GI tract of pigs and after enrichment

We employed in vivo and in vitro enrichment procedures with the goal to isolate bacteria capable of metabolizing gluten peptides (Fig. 5.1). Pigs were fed a basal diet supplemented with 20% gluten for 16 weeks to enrich for intestinal bacteria in vivo. In addition, an enrichment step was performed in a defined growth medium (GPE) containing the toxic gluten peptide LQLQPFPQPQLPYPQPQL, but not the individual amino acids included in the peptide. In total, 140 isolates were obtained, 87 directly from the SI contents and 53 isolates from the enrichment (Fig. 5.1). All isolates were typed by RAPD and 96 isolates with distinct patterns were taxonomically identified by 16S rRNA gene sequence analysis.

In accordance with the microbiota composition of the proximal gut of pigs (Leser *et al.* 2002), the strains isolated directly from the SI contents, belonged to the genera *Lactobacillus* (33%), *Clostridium* (20%), *Escherichia* (18%) and *Streptococcus* (13%) with the remaining belonging to *Bacillus* (10%) and other genera (6%). After the GPE step, lactobacilli accounted for 57% of the strains isolated, with the majority of the isolates belonging to the species *L. ruminis and L. amylovorus*. Other genera included *Enterococcus* (40%) and *Streptococcus* (3%). Therefore, it appears that the *in vitro* enrichment favored the growth of LAB, particularly lactobacilli and enterococci. Comparison of the bacterial populations in the GPE media with and without the peptide by DGGE confirmed the enrichment of several bacterial species, including *Lactobacillus* and *Enterococcus*, through the 18-mer (Fig. 5.2a). Quantification via UHPLC-MS/MS-MRM also revealed that the peptide was gradually degraded during the enrichment step (Fig.5.2b). Interestingly, peptide concentration showed a significant negative correlation ($p < 0.005$) with the staining intensity of a DGGE fragment corresponding to *L. amylovorus* (Fig. 5.2c).

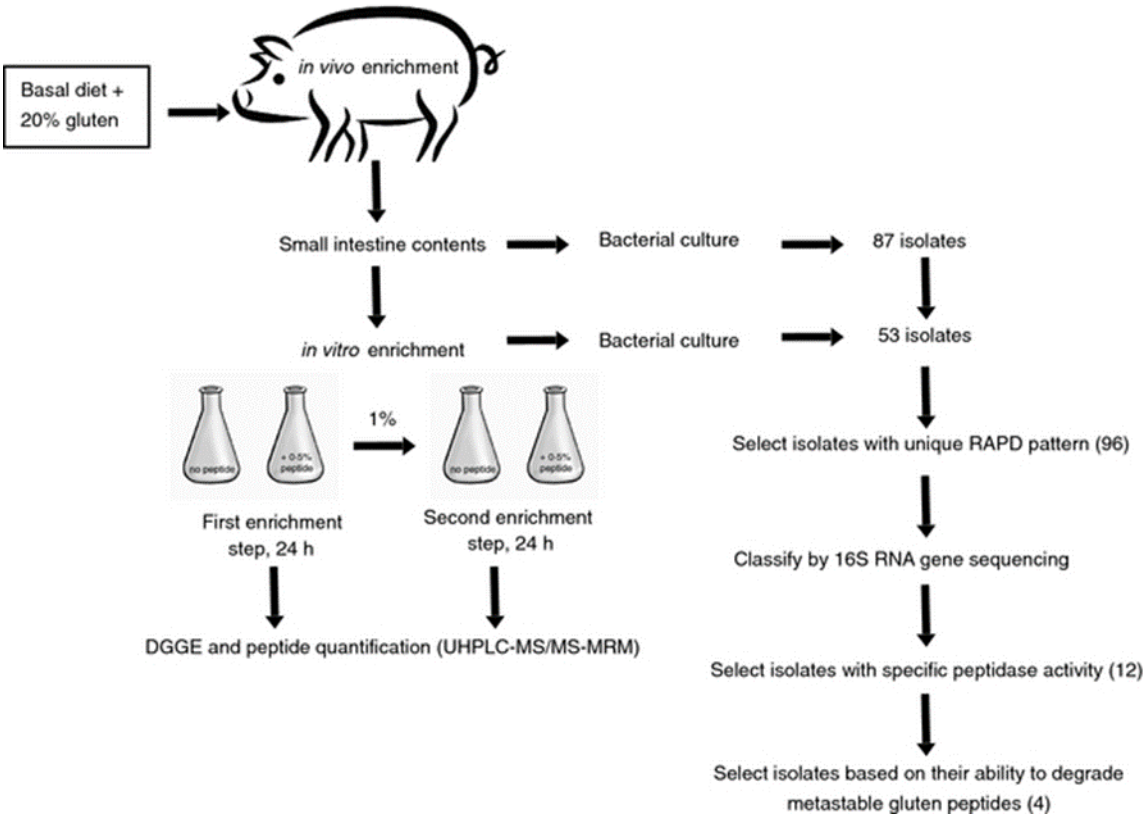

Figure 5.1 Experimental design

In vivo (in pigs) and in vitro enrichment procedures used in this study to select gluten-degrading bacteria adapted to the proximal GI tract. Number of strains selected from each step is shown in brackets.

5.3.2 Screening for isolates with peptidase activity

All 96 unique strains based on RAPD analysis were screened for the production of specialized peptidases known to degrade immunotoxic gluten peptides (Piper, Gray and Khosla 2004; De Angelis *et al.* 2010). PepN and PEP were the main enzymatic activities observed among the strains, while PepI activity was lower but also detected in a number of isolates (Fig. 5.S1). Based on this analysis, 12 isolates with the highest overall activity were selected for in depth characterization.

5.3.3 Degradation of peptides involved in the etiology of celiac disease

To evaluate the potential of the selected 12 strains to detoxify gluten epitopes, we determined the degradation of the metastable 33-mer, 16-mer, and 17-mer peptides, which have been shown to be involved in the etiology of celiac disease (Shan et al. 2002; Tye-Din et al. 2010). Two different quantitative ELISA assays, in addition to UHPLC-MS/MS-MRM analysis, were used to quantify the peptides before and after 24 hour incubation with the bacterial strains. This analysis revealed that several of the strains degraded the three peptides, but differed markedly in their hydrolytic activity towards the immunogenic peptides (Fig.5.S2-S4). Based on the combined results from these assays, four gluten peptide degrader (GPD) strains (*L. amylovorus* GPD2, *L. johnsonii* GPD6*, L. ruminis* GPD9*,* and *L. salivarius* GPD12) with the highest rates of degradation were selected.

The selected GPD isolates were characterized in depth for their ability to hydrolyze the gluten peptides as individual strains and as a mixture (Fig. 5.3). The RIDASCREEN® ELISA was used to measure degradation, as the R5 antibody detected all gluten peptides studied (Table S3). As shown in Figure 5.3, the findings were in general agreement with those from the initial screen, and all GPD isolates hydrolyzed the gluten peptides at different rates. Specifically, the hydrolytic actions of the strains *L. salivarius* GPD12 and *L. amylovorus* GPD2 were the most effective in

degrading the 33-mer and the 17-mer peptides, while the 16-mer was more effectively degraded by the strains *L johnsonii* GPD6 and *L. ruminis* GPD9. The GPD strains were mixed to test whether the diverse proteolytic specificity shown could be combined to achieve a synergistic effect. The amount of degradation achieved was typical of the strain that best degraded that particular peptide. However, the mixture of strains produced an overall more uniform degradation of all three peptides than the individual strains GPD2, GPD6 and GPD9, and outperformed the strain GPD12 in degradation of the 16-mer.

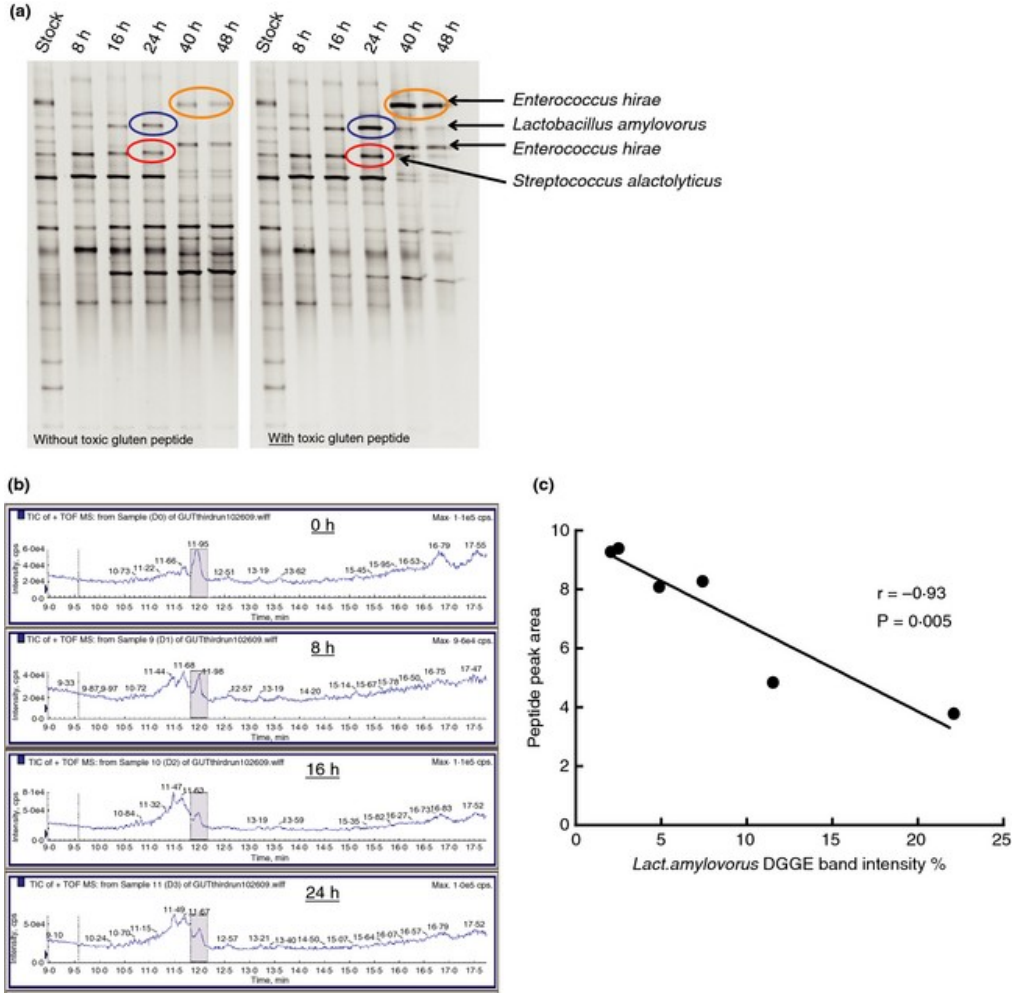

Figure 5.2 Microbiological and analytical characterization of the bacterial enrichment cultures

(a) DGGE during incubation without (left) and with (right) the 18-mer peptide. Corresponding bands are indicated by colored circles. (b) UHPLC-MS/MS-MRM analysis of the first 24 hours of enrichment. Peak corresponding to the 18-mer is indicated with a blue box. (c) Correlation between peak area and the DGGE band intensity of *L. amylovorus.*

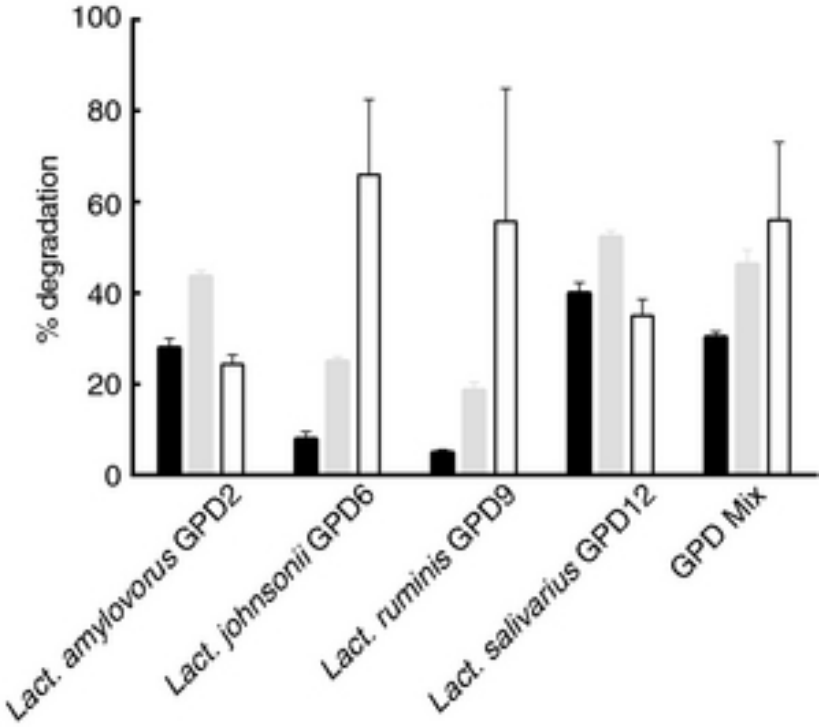

Figure 5.3 Degradation of the gluten peptides by the selected gluten peptide degrader GPD strains

33-mer (black), 17-mer (grey), 16-mer (white). Bars represent the average percent degradation (n=3 ± SD) after 24 hours determined from peptide amounts in test and control samples quantified using the RIDASCREEN® ELISA.

5.3.4 Characterization of cleavage fragments and proteolytic specificity

To evaluate to which degree the GPD strains removed T-cell stimulating epitopes from peptides, the amino acid sequences of the bacterial hydrolysis fragments were determined. According to the LC-MS/MS results, all the GPD strains partially hydrolyzed the highly resistant 33-mer mainly by targeting post proline and post glutamine bonds (Table S5). But more importantly, at least one cleavage site was found in all of the six overlapping epitopes producing a variety of peptide fragments with a reduced number of immunodominant T-cell epitopes (Table 6.1 and Fig. 5.4). As shown in Figure 5.4, hydrolysis of the 33-mer peptide was strain-dependent and cleavage patterns were in general accordance with enzyme production levels by each GPD strain. Specifically, the PepN from *L. amylovorus* GPD2 (isolate 50 in Fig. 5.S1) targeted the bonds at the N-terminal of the peptide, generating fragments of 8 to 28 amino acids (Fig. 5.4b). Three short fragments, lacking 9 amino acid T-cell stimulatory core epitopes, were also produced. Hydrolytic actions of *L. johnsonii* GPD6 (isolate 19 in Fig. 5.S1) reflected the endoproteolytic activity of PEP in combination with PepN cleavage, producing medium sized fragments with one to 5 epitopes, and several small fragments (8 – 13 aa) (Fig. 5.4c). *L. ruminis* GPD9 (isolate 44 in Fig 5.S1), a high producer of PepN and PEP, cleaved at both ends of the 33-mer peptide, leaving a few short peptide fragments absent of known T-cell stimulating epitopes, and several other fragments containing as many as five epitopes (Fig. 5.4d). Hydrolysis by the strain *L. salivarius* GPD12 (isolate 59 in Fig 5.S1) produced the highest reduction of the native form of the 33-mer generating the truncated peptide PQPQLPYPQPQLPYPQPQLPYPQPQP. Reflective of PepN activity, hydrolysis occurred at the N-terminus of the peptide. Other degradation fragments ranged from 7 to 28 amino acids, the majority containing two or more epitopes (Fig. 5.4d). The hydrolytic activity of the GPD mixture produced truncated 33-mer peptide fragments ranging from14 to 26 aa. Smaller fragments (< 8 aa) were not detected (Fig. 5.4e).

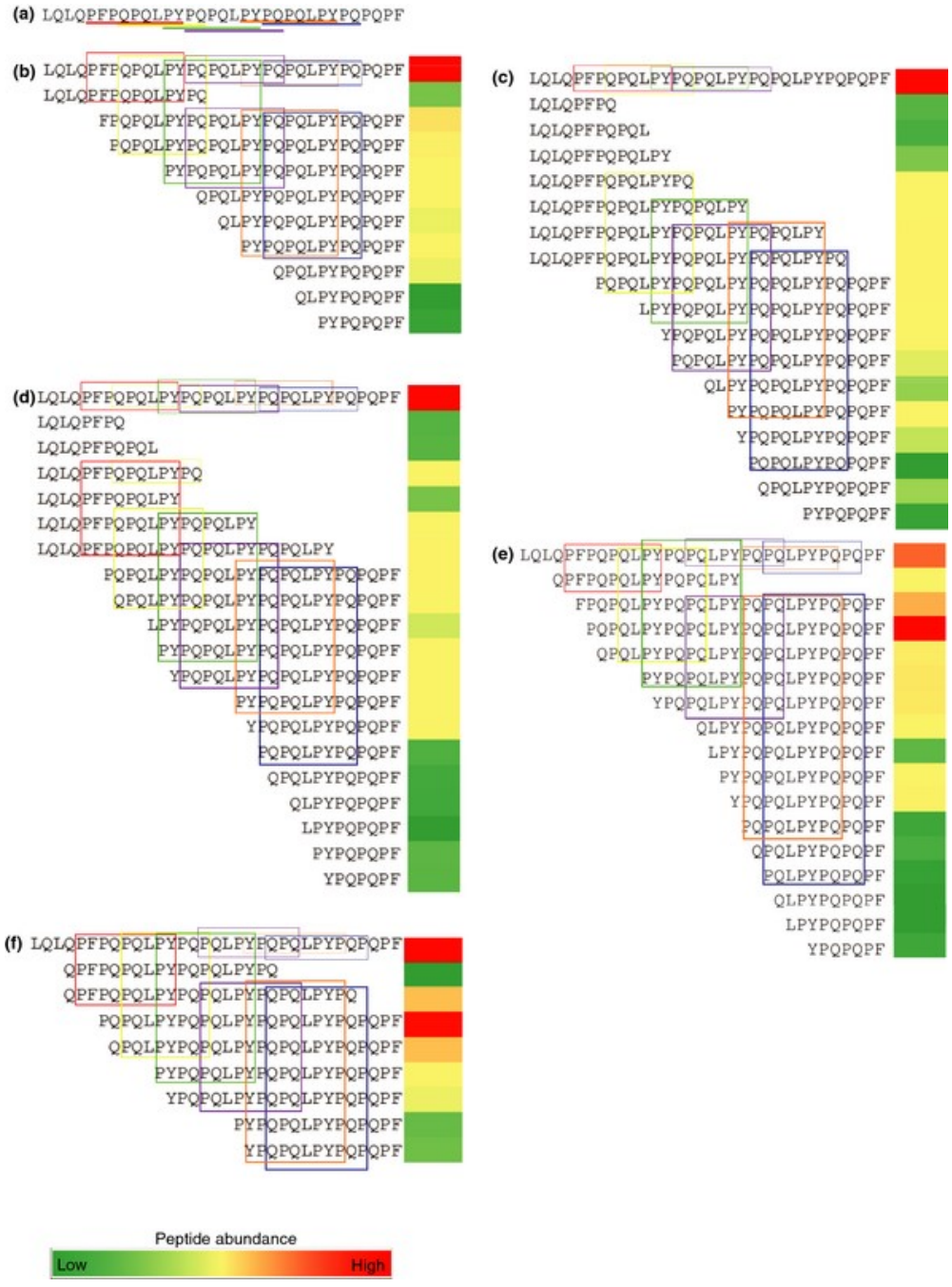

Figure 5.4 Characterization of peptide degradation by LC-MS/MS

(a) Peptide sequence of the 33-mer with immunostimulatory T cell epitopes underlined (Sollid et al. 2012). Peptide fragments after hydrolysis by (b) L. *amylovorus* GPD2, (c) *L. johnsonii* GPD6, (d) *L. ruminis* GPD9, (e) *L. salivarius* GPD12, and (f) the GPD mix. Relative fragment abundance is represented by a colored scale and the remaining T-cell epitopes are indicated in colored boxes same as (a).

Hydrolysis of the 17-mer peptide was less proline specific and primarily targeted the bond after the Q5 residue, which is located outside the 9 amino acid epitope core. Fragments generated ranged from 11 to 14 amino acids (Table S6) and only *L. salivarius* GPD12 cleaved the bonds P↓F and P↓W located within the two overlaping epitopes (Table 6.1).The 16-mer was hydrolyzed into smaller fragments of 9 or 10 residues. The most common cleavage site was between the F5 and the P6 residues generating the fragment PQPQLPYPQPQ. Hydrolysis with *L. amylovorus* GPD2 and *L. salivarius* GPD12 also generated the fragment QPQLPYPQPQ (Table S7). All the GPD strains with the exception of *L. johnsonii* GPD6, which cleaved at only one of the epitopes, hydrolyzed bonds contained within the two overlapping epitopes of the 16-mer peptide (Table 6.1). In general, peptide degradation by the GPD mix reflected the hydrolysis produced by the individual strains with the exception of the cleavage site between Q29 and P30 of the 33-mer which was not produced by any of the GPD isolates alone (Table 6.1).

Table 5.1 Cleavage sites in the immunotoxic peptides by bacterial strain or strain mixture

	33mer* LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	17mer* QPQQPFPQPQQPF	16mer* QLQPFPQPQLPY
L. amylovorus GPD2	LQLQP⬇F⬇PQPQL⬇PYP⬇Q⬇P↓QL⬇PYP↓QP↓QL↓PYPQP QPF	QPQ↓QPFPQPQQPFP WQP	QLQPF↓P⬇QPQLPY PQPQ
L. johnsonii GPD6	LQLQPF⬇PQ↓P⬇QL↓P⬇Y⬇PQ⬇P↓Q⬇L⬇PY↓P↓QPQL⬇P Y⬇PQPQPF	QPQ⬇QPFPQPQQPF PWQ↓P	QLQPF↓PQPQLPYPQ PQ
L. ruminis GPD9	LQLQPF⬇P⬇Q↓PQ↓L⬇P⬇Y↓PQ⬇PQL⬇P⬇Y↓P↓Q↓P↓Q↓L ↓P↓Y⬇PQPQPF	QPQQPFPQPQQPFP WQP	QLQPFPQPQ↓LPYPQ PQ
L. salivarius GPD12	LQL⬇QP⬇F⬇P⬇QPQL⬇P⬇YPQP⬇Q↓L⬇P⬇Y↓P↓Q↓P↓Q↓ LP↓YPQPQPF	QPQ⬇QP↓FPQPQQP FP↓WQP	QLQPF⬇P⬇Q↓PQLP YPQPQ
GPD mix†	LQL⬇QPF⬇P⬇QPQL↓P↓YPQPQL↓P↓YPQ↓PQLPYPQ⬇PQ PF	QPQ⬇QP↓FPQPQQP FPWQP	QLQPF⬇P↓QPQLPY PQPQ

*T-cell stimulatory epitopes (Tye-Din et al. 2010; Sollid et al. 2012) are indicated with a black line.
⬇ Arrows indicate major cleavage sites
↓Arrows indicate less efficiently cleaved sites.
† Combination of all four isolates

5.3.5 Comparison with commercial probiotic strains

Peptide degradation abilities of strains obtained during this study were compared with those of bacteria commercially used as probiotics using the RIDASCREEN® ELISA as described above. In general, the probiotic strains differed widely in their ability to degrade the peptides and some (*B. animalis* BB-12, *L. reuteri* ATCC 55730) showed very little or no hydrolytic activity. *L. rhamosus* GG (all peptides), *L. casei* ATCC 334 (33-mer and 16-mer), *L. plantarum* 299v (33-mer and 17-mer) and *L. acidophilus* NCFM (16-mer), showed similar degradation to the GPD strains (Table S4). However, the mixture of GPD isolates outperformed the eight members of the probiotic preparation VLS#3 in the hydrolysis of the immunogenic peptides (Table S4).

5.4 Discussion

The objective of this study was to characterize the ability of bacteria from the proximal GI tract of pigs, to degrade toxic gluten epitopes in order to identify candidates for probiotic applications. Our approach involved *in vivo* and *in vitro* enrichment procedures to select for gluten degrading bacteria. Pigs are physiologically similar to humans (Heinritz, Mosenthin and Weiss 2013) but harbor higher numbers of LAB in the proximal GI tract (Castillo *et al.* 2007). These animals are an excellent model for humans and their use allowed to feed high amounts of gluten to stimulate gluten-metabolizing bacteria in the small intestine, a region that harbors only very low numbers of bacteria in humans (Walter 2008). Analytical and microbiological characterization of the bacterial population dynamics during and after the *in vitro* enrichment, suggest lactobacilli had a competitive advantage over other intestinal bacteria, conferred by their ability to utilize metastable gluten peptides to obtain nitrogen.

Our extensive screening approach further confirmed this hypothesis as the strains with the highest gluten-degrading abilities belonged to the species *L. amylovorus*, *L. johnsonii*, *L. ruminis*, and *L. salivarius*. These strains degraded the metastable peptides 33-mer, 16-mer, and 17-mer, which have been shown to be relevant for the immune response in celiac disease (Shan et al.

2002; Tye-Din et al. 2010). Our findings are in accordance to those of Caminero et al. (2014) in that we also identified lactobacilli as the dominant gluten degrading bacteria in the GI tract. Interestingly, both studies have identified *L. ruminis* and *L. amylovorus* as the major gluten degraders, supporting the role of these species in gluten degradation.

Particular aspects of the physiology of lactobacilli could assist in the degradation of gluten. LAB produce various peptidases aimed to obtain amino acids from the nutrient-rich environments where they typically inhabit (Fernández and Zúñiga 2006; Makarova *et al.* 2006). Their limited biosynthetic metabolism is compensated by an array of hydrolytic enzymes and transport systems (Makarova *et al.* 2006). To satisfy their nitrogen needs, extracellular and cell-wall bound proteases cleave proteins into oligopeptides that are transported across the membrane to be hydrolyzed by intracellular peptidases into free amino acids (Christensen *et al.* 1999). The proteolytic enzymes expressed by lactobacilli are capable of cleaving proline bonds, which was evidenced by our findings and their ability to utilize the proline-rich protein casein (Christensen *et al.* 1999). In fact, various strains of LAB degrade toxic gluten peptides during sourdough fermentation (De Angelis *et al.* 2006, 2010; Gobbetti *et al.* 2007; Rizzello *et al.* 2007).

The degree by which the GPD isolates degrade immunogenic peptides appears to be peptide-dependent, and it is likely to involve both extracellular proteolysis and peptide uptake. Specifically, hydrolysis of the 33-mer was the lowest among the three peptides when analyzed by ELISA. However, LS-MS/MS analysis revealed the production of several medium-sized and short fragments that were detected in low abundance. On the contrary, significant reduction of the 16-mer and 17-mer peptides was detected by ELISA, but fewer and only slightly truncated fragments were produced. This trend was observed in all the GPD isolates. Therefore, it is possible that the longer 33-mer peptide was target of extracellular hydrolysis and that intact or slightly truncated 17-mer and 16-mer peptides were transported across the membrane to be cleaved in the cytoplasm. It has been well established that some LAB are capable of transporting proline rich oligopeptides of various lengths through the cell membrane (Berntsson *et al.* 2009) and several

species of lactobacilli, encode for these transporters in their genomes (Liu *et al.* 2010). However, more functional studies are required to determine how the GPD strains remove the metastable peptides.

It is important to point out that, although the GPD strains can hydrolyze metastable gluten peptides, the degradation products are likely still immunogenic. However, partial cleavage, especially at the proline level could potentially reduce peptide resistance to hydrolysis by digestive enzymes, and it might constitute a beneficial trait if probiotic strains are used in combination with gluten-degrading enzymes in clinical applications (Tye-Din et al. 2010; Siegel et al. 2012; Tack et al. 2013; Lähdeaho et al. 2014). *Lactobacillus* strains might have synergistic activities with enzymes to completely destroy the immunogenic epitopes by complementing their cleavage range. For example, the GPD stains mainly cleaved at the N and C terminus of the peptides leaving immunogenic internal fragments intact. Combination of the GPD strains with enzymes that preferably cleave at internal bonds (Stepniak *et al.* 2006) could be act synergistically to completely destroy peptide epitopes. Additionally, fragments generated from partial peptide hydrolysis could be subsequently cleaved by enzymes that show preference for short immunogenic substrates (Shan *et al.* 2004). Further research is required to determine if combinations of enzymes and GPD bacteria successfully eliminate peptide immunogenicity. The information presented in this study can hereby serve as a guide for the selection of strains to be combined with specific gluten-degrading probiotics and enzymes.

This work, together with recently published reports (Laparra and Sanz 2010; Zamakhchari *et al.* 2011; Fernandez-Feo *et al.* 2013; Caminero *et al.* 2014) provides a basis for the selection of probiotic strains as a potential treatment for celiac patients (de Sousa Moraes *et al.* 2014). Delivery of the probiotic strains could be achieved in the form of capsules administered before and after a meal to assure the presence of lactobacilli at the time of gluten exposure. Probiotic lactobacilli (including strains from pigs) persist in the human gut for a number of days (Jacobsen *et al.* 1999) and several LAB strains haven been successfully used as delivery vehicles of mucosal

therapeutic biomolecules (Daniel *et al.* 2011). These findings are encouraging in that they suggest that lactobacilli could be delivered, in a functional state, to the relevant locations in the gut. Given the incomplete degradation of the immunotoxic peptides by the GPD strains identified in our study, the use of a probiotic treatment alone is unlikely to allow patients to consume significant amounts of gluten. However, probiotics could potentially aid to control the detrimental effects from accidental exposure and contamination of *bona fide* gluten-free products, and they could be combined with gluten degrading enzymes with synergistic activity. However, research is needed to determine the gluten degrading potential lactobacilli *in vivo*, and to develop therapeutic formulations and dosing regimens for clinical applications. Probiotic lactobacilli could be used in human clinical research without extensive hurdles, as these bacteria are generally harmless and well tolerated (even if consumed in high numbers), and various strains have a “generally recognized as safe” status (GRAS) (Wells and Mercenier 2008). In this study, we showed that several commercially available probiotic strains (*L. rhamnosus* GG, *L. casei* 334, and *L. plantarum* 299v), that could be readily applied, also degrade the immunotoxic peptides to some extent. However, the four porcine stains identified during our strain selection might have advantages to human colonic (Laparra and Sanz 2010; Caminero *et al.* 2014), oral (Zamakhchari *et al.* 2011; Fernandez-Feo *et al.* 2013), and genetically engineered bacteria (Alvarez-Sieiro *et al.* 2014) in that they are naturally adapted to the conditions of the small intestine, and therefore are likely more physiologically active and functional. However, ultimately, only clinical trials can determine which strains work best in humans.

5.5 Supplementary material

Table S2 Sample dilution and standard curve for RIDASCREEN® ELISA

Peptide	Sample dilution	Std. 1 (µg ml^{-1})	Std. 2 (µg ml^{-1})	Std. 3 (µg ml^{-1})	Std. 4 (µg ml^{-1})	Std. 5 (µg ml^{-1})
17-mer	1:100	10	3.3	1.1	0.4	0.1
16-mer	1:7	143	47.7	15.9	5.3	1.7
33-mer	1:5	200	66.7	22.2	7.4	2.5

Dilutions were prepared in the sample dilution buffer provided with the kit

Table S3 Sample dilution and standard curve for the GlutenTox® ELISA

Peptide	Sample dilution	Std. 1 (µg ml^{-1})	Std. 2 (µg ml^{-1})	Std. 3 (µg ml^{-1})	Std. 4 (µg ml^{-1})	Std. 5 (µg ml^{-1})
16-mer	1:5000	200	100	50	25	12.5
33-mer	1:5000	200	100	50	25	12.5

Dilutions were prepared in the sample dilution buffer provided with the kit

Table S3 Epitopes recognized by the ELISA tests.

Peptide	Amino acid sequence
33 mer.	LQ**LQPFP***QPQLPY*P*QPQLPY*P**QPQLPY**PQPQPF
17 mer.	QP**QQPFP**QP**QQPFP**WQP
16 mer.	Q**LQPFP***QPQLPY*PQPQ

Epitopes recognized by the monoclonal antibodies R5 (RIDASCREEN®) and G12 (GlutenTox®) are shown in **bold** or *italicized,* respectively

Table S4 Percent peptide degradation by the GPD and commercial probiotic strains

	L. amylovorus GPD2	*L. johnsonii* GPD6	*L. ruminis* GDP9	*L. salivarius* GDP12	*L. plantarum* 299v	*L. casei* ATCC 334	*L. acidophilus* NCFM	*B. animalis* BB-12	*L. reuteri* ATCC 55730	*L.rhamnosus* GG	GPD mix	VSl#3
33-mer	28.0 ± 3.5	8 ± 2.6	5.0 ± 1.0	40.0 ± 4.0	41.0 ± 23	19.0 ± 7.5	0 ± 0	5.3 ± 9.2	2.7 ± 4.6	26 ± 21	30.0 ± 2.1	4.5 ± 2.6
17-mer	44.0 ± 2.1	25 ± 1.7	19 ± 3.1	52 ± 2.3	37 ± 1.2	0.67 ± 0.58	0.67 ± 1.2	1.7 ± 2.9	0.67 ± 0.58	53.0 ± 13.0	43.5 ± 5.5	35.0 ± 10.2
16-mer	24.0 ± 2.1	66.0 ± 16.0	56.0 ± 29.0	35.0 ± 3.6	0.67 ± 0.58	41 ± 18	48 ± 37	1.7 ± 1.2	0.67 ± 0.58	25 ± 6.4	65.0 ± 17.1	19 ± 4.2

Table S7. MS-MS data from 16-mer hydrolysis by each strain

strain	start aa	sequence	area
L. amylovorus GDP2	1	QPQQPFPQPQQPFPWQP	1.34E+08
	4	QPFPQPQQPFPWQP	4.85E+06
L. johsonii GDP6	1	QPQQPFPQPQQPFPWQP	2.05E+08
	1	QPQQPFPQPQQPFPWQ	1.01E+06
	4	QPFPQPQQPFPWQP	1.37E+06
L. ruminis GDP9	1	QPQQPFPQPQQPFPWQP	2.24E+08
L. salivarius GDP12	1	QPQQPFPQPQQPFPWQP	9.36E+07
	4	QPFPQPQQPFP	2.23E+05
	4	QPFPQPQQPFPWQP	1.71E+08
	6	FPQPQQPFPWQP	1.64E+06
GDP mix	1	QPQQPFPQPQQPFPWQP	1.00E+08
	4	QPFPQPQQPFPWQP	4.51E+06
	6	FPQPQQPFPWQP	4.04E+06

Table S6. MS-MS data from 16-mer hydrolysis by each strain

	start aa	sequence	peak area
L. amylovorus GDP2	1	QLQPFPQPQLPYPQPQ	2.57E+08
	7	PQPQLPYPQPQ	2.79E+05
	8	QPQLPYPQPQ	3.25E+05
L. johsonii GDP6	1	QLQPFPQPQLPYPQPQ	1.32E+08
	7	PQPQLPYPQPQ	1.40E+05
L. ruminis GDP9	1	QLQPFPQPQLPYPQPQ	1.79E+08
	1	QLQPFPQPQ	1.35E+05
L. salivarius GDP12	1	QLQPFPQPQLPYPQPQ	3.40E+08
	7	PQPQLPYPQPQ	3.52E+05
	8	QPQLPYPQPQ	3.31E+05
	9	PQLPYPQPQ	1.29E+05
GDP MIX	1	QLQPFPQPQLPYPQPQ	2.91E+08
	7	PQPQLPYPQPQ	3.25E+05
	8	QPQLPYPQPQ	2.84E+05

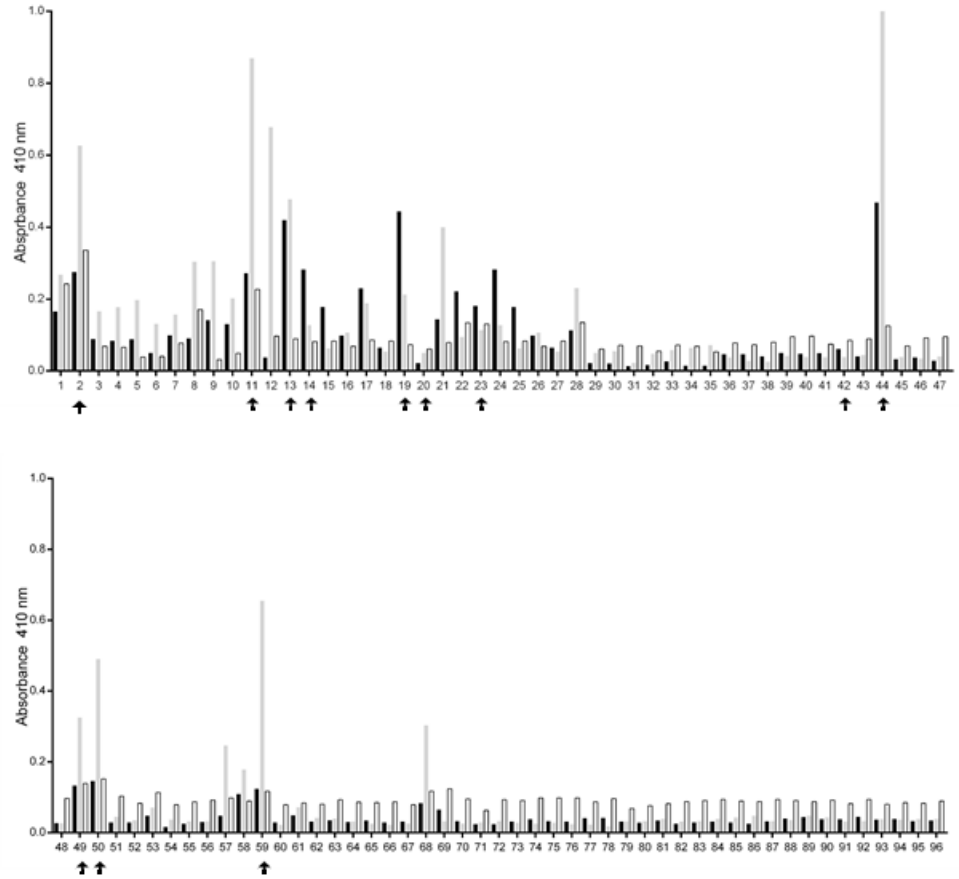

Fig. S1 Specialized peptidase production measured as the release of *p*-nitroaniline from chromogenic substrates. Bars represent absorbance at 410 nm after 4 hours. Prolyl endopeptidase (black), aminopeptidase N (grey), and proline iminopeptidase (white). Isolates selected for gluten peptide degradation experiments are indicated with an arrow (⬆)

Table S5. MS-MS data from 33-mer hydrolysis by each strain

	start aa	sequence	area
L amylvorus GDP2	1	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	1.67E+08
	1	LQLQPFPQPQLPYPQ	2.68E+06
	6	FPQPQLPYPQPQLPYPQPQLPYPQPQPF	4.03E+05
	7	PQPQLPYPQPQLPYPQPQLPYPQPQPF	9.86E+06
	12	PYPQPQLPYPQPQLPYPQPQPF	2.77E+06
	15	QPQLPYPQPQLPYPQPQPF	1.02E+06
	17	QLPYPQPQLPYPQPQPF	6.17E+05
	19	PYPQPQLPYPQPQPF	5.69E+05
	22	QPQLPYPQPQPF	5.91E+05
	24	QLPYPQPQPF	5.72E+05
	26	PYPQPQPF	2.18E+05
L. johnsonii GDP6	1	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	2.52E+08
	1	LQLQPFPQ	3.76E+05
	1	LQLQPFPQPQL	3.40E+05
	1	LQLQPFPQPQLPY	4.46E+05
	1	LQLQPFPQPQLPYPQ	9.54E+05
	1	LQLQPFPQPQLPYPQPQLPY	9.56E+05
	1	LQLQPFPQPQLPYPQPQLPYPQPQLPY	2.51E+06
	1	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQ	2.45E+06
	7	PQPQLPYPQPQLPYPQPQLPYPQPQPF	2.24E+06
	11	LPYPQPQLPYPQPQLPYPQPQPF	6.62E+05
	13	YPQPQLPYPQPQLPYPQPQPF	1.41E+06
	14	PQPQLPYPQPQLPYPQPQPF	5.90E+05
	17	QLPYPQPQLPYPQPQPF	4.83E+05
	19	PYPQPQLPYPQPQPF	7.12E+05
	20	YPQPQLPYPQPQPF	5.49E+05
	21	PQPQLPYPQPQPF	2.51E+05
	22	QPQLPYPQPQPF	4.95E+05
	26	PYPQPQPF	2.84E+05
L. ruminis GDP9	1	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	2.42E+08
	1	LQLQPFPQ	3.47E+05
	1	LQLQPFPQPQL	3.58E+05
	1	LQLQPFPQPQLPYPQ	9.46E+05
	1	LQLQPFPQPQLPY	4.57E+05
	1	LQLQPFPQPQLPYPQPQLPY	9.03E+05
	1	LQLQPFPQPQLPYPQPQLPYPQPQLPY	2.33E+06
	7	PQPQLPYPQPQLPYPQPQLPYPQPQPF	2.02E+06
	8	QPQLPYPQPQLPYPQPQLPYPQPQPF	8.49E+05
	11	LPYPQPQLPYPQPQLPYPQPQPF	6.55E+05
	12	PYPQPQLPYPQPQLPYPQPQPF	1.21E+06
	13	YPQPQLPYPQPQLPYPQPQPF	1.68E+06
	19	PYPQPQLPYPQPQPF	9.05E+05
	20	YPQPQLPYPQPQPF	1.08E+06
	21	PQPQLPYPQPQPF	3.27E+05
	22	QPQLPYPQPQPF	2.63E+05
	24	QLPYPQPQPF	2.58E+05
	25	LPYPQPQPF	1.57E+05
	26	PYPQPQPF	3.69E+05
	27	YPQPQPF	3.65E+05
L. salivarius GDP12	1	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	6.92E+06
	4	QPFPQPQLPYPQPQLPY	1.01E+06
	6	FPQPQLPYPQPQLPYPQPQLPYPQPQPF	3.84E+06
	7	PQPQLPYPQPQLPYPQPQLPYPQPQPF	1.39E+07
	8	QPQLPYPQPQLPYPQPQLPYPQPQPF	1.39E+06
	12	PYPQPQLPYPQPQLPYPQPQPF	1.54E+06
	13	YPQPQLPYPQPQLPYPQPQPF	1.51E+06
	17	QLPYPQPQLPYPQPQPF	1.01E+06
	18	LPYPQPQLPYPQPQPF	5.57E+05
	19	PYPQPQLPYPQPQPF	1.05E+06
	20	YPQPQLPYPQPQPF	1.16E+06
	21	PQPQLPYPQPQPF	3.87E+05
	22	QPQLPYPQPQPF	4.59E+05
	23	PQLPYPQPQPF	3.27E+05
	24	QLPYPQPQPF	2.87E+05
	25	LPYPQPQPF	2.95E+05
	27	YPQPQPF	3.92E+05
GDP mix	1	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	9.67E+06
	4	QPFPQPQLPYPQPQLPYPQ	2.80E+05
	4	QPFPQPQLPYPQPQLPYPQPQLPYPQ	2.64E+06
	7	PQPQLPYPQPQLPYPQPQLPYPQPQPF	8.88E+06
	8	QPQLPYPQPQLPYPQPQLPYPQPQPF	2.64E+06
	12	PYPQPQLPYPQPQLPYPQPQPF	1.23E+06
	13	YPQPQLPYPQPQLPYPQPQPF	1.18E+06
	19	PYPQPQLPYPQPQPF	6.67E+05
	20	YPQPQLPYPQPQPF	7.14E+05

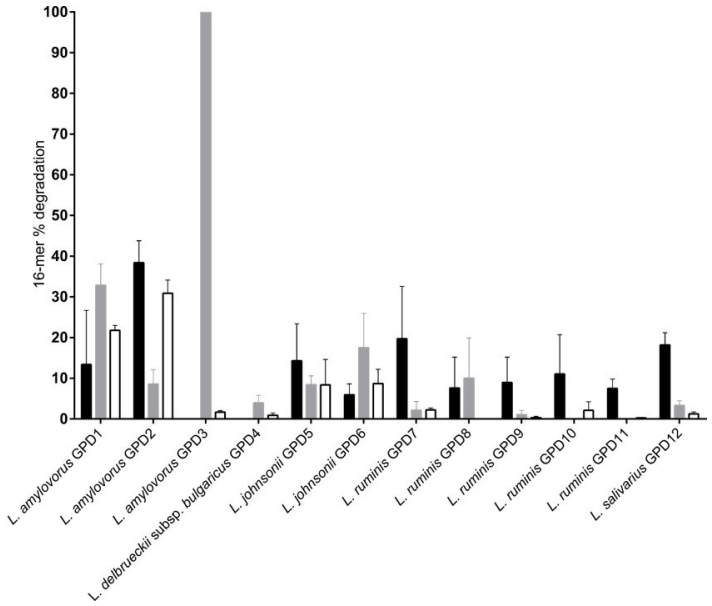

Fig. S2. 16-mer degradation by the selected isolates. Bars represent the percent degradation quantitated by (black) RIDASCREEN® ELISA, (white) GlutenTox® ELISA, and (grey) and UHPLC-MS/MS-MRM. Data is presented as the average (n=2 ± STD) Absence of bar indicates no degradation detected.

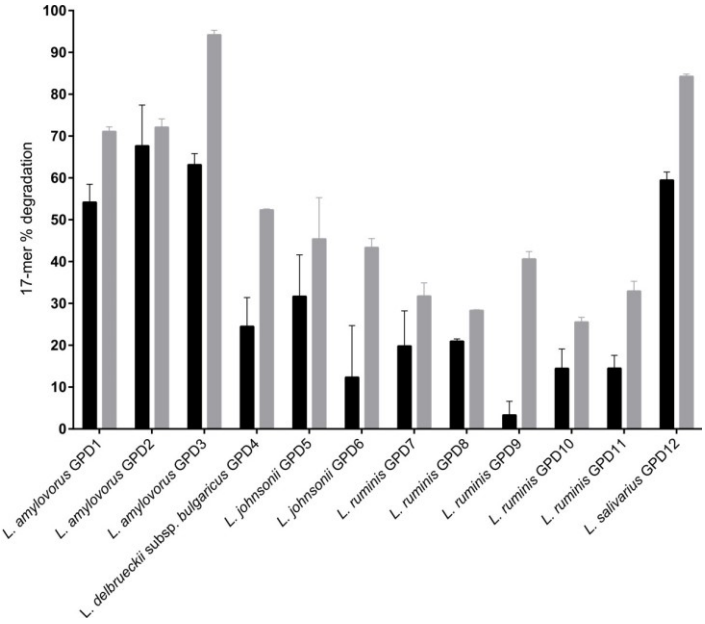

Fig. S3 17-mer degradation by the selected isolates. Bars represent the percent degradation quantitated by (black) RIDASCREEN® ELISA, (white) GlutenTox® ELISA, and (grey) and UHPLC-MS/MS-MRM. Data is presented as the average (n=2 ± STD) Absence of bar indicates no degradation detected.

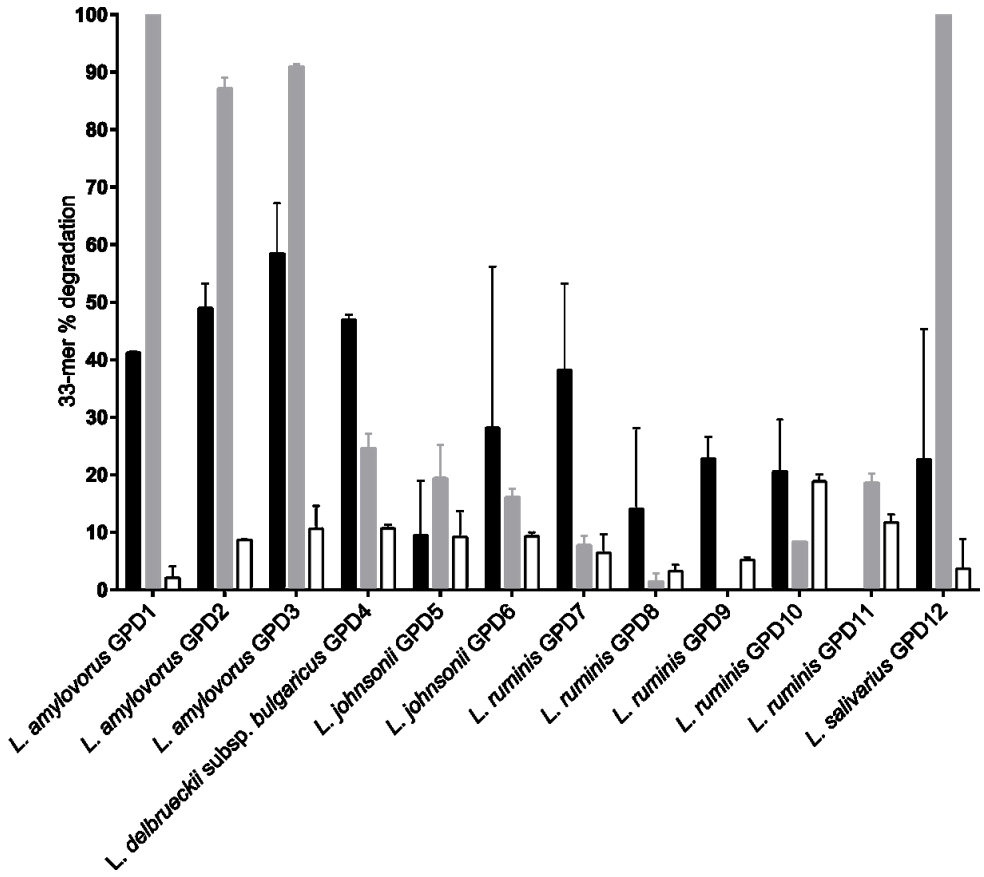

Fig S4 33-mer degradation by the selected isolates. Bars represent the percent degradation quantitated by (black) RIDASCREEN® ELISA, (white) GlutenTox® ELISA, and (grey) and UHPLC-MS/MS-MRM. Data is presented as the average (n=2 ± STD) Absence of bar indicates

5.6 References

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6. Chapter six: Conclusions, future directions and implications

6.1 Conclusions and future directions

The phylogenomic framework presented in **Chapter one** allowed inferences regarding the natural history and lifestyle of lactobacilli and proposes a model for the evolution of distinct lifestyles within the genus, which range from free-living to strictly host-adapted and symbiotic species. Lifestyles of lactobacilli relate to their phylogeny and members of the same phylogenetic group or clade typically share important metabolic traits as well as characteristic aspects of their lifestyle. Many lactobacilli remain free-living, or adopted a nomadic lifestyle to use animal hosts for dispersal. Different groups in the genus adapted complex lifestyles that allowed them to colonize diverse animal hosts, ranging from insects to humans, with a different degree of host-specificity (Fig 1.6).

Chapter two explored the consequences of the evolution of *L. reuteri* with different vertebrate species. Findings from this work established that *L. reuteri* evolved jointly not only with mice but also chicken, and that this process of co-evolution resulted in specialization. Those strains that evolved jointly with their hosts showed elevated fitness when compared with strains that share no natural history with that same host.

Work in **Chapter four** revealed that the ability of human strains to cross-feed from 1,2 propanediol (1,2 PD) could be relevant for their competitiveness in the gut. Rhamnose, one of the precursors of 1,2 PD reaches the gut through pectin and hemicellulosic polysaccharides present in plant fibers. Given that modern diets are significantly low in plant fibers (Deehan and Walter, 2016) a reduced amount of rhamnose, and consequentially of 1,2 PD, could mean that the conditions in which *L. reuteri* evolved in humans are no longer existent and would explain findings in **Chapter two.**

As reviewed in **Chapter one**, a common strategy by which lactobacilli maintain host-specificity is by adhering to the host's tissue (Fig 1.4). Rodent strains of *L. reuteri* adhere to the mouse forestomach by forming biofilms, a mechanism that is restricted to rodent strains (Frese et al. 2013). Work in **Chapter three** characterized a two component system (TCS70529-30) which is highly conserved in rodent strains and had been hypothesized to have a regulatory role in biofilm formation (Frese *et al.* 2011). Findings from *in vivo* and *in vitro* experiments confirmed that TCS70529-30 has a role in biofilm formation, most likely by regulating genes involved in the development of the exopolymeric matrix. Deletion of the individual genes of the two component system resulted in highly disparate phenotypes, with histidine kinase mutants producing robust biofilms, while response regulator mutants produced fragile biofilms (Fig 3.2-3.7). Future studies are thus required to fully elucidate the role of TCS70529-30 in biofilm formation. Efforts to determine the transcriptional targets of TCS70529-30 are currently underway.

One powerful characteristics of *L. reuteri* as model species to study adaptation to the vertebrate gut, is that strains from different hosts differ at the genetic level. These differences often correlate with the conditions encountered in the gut of each corresponding host (Frese *et al.* 2011). For example, contrary to rodents strains, human isolates conserve a 58-gene cluster encoding a 1,2 propanediol dehydratase PduCDE. **Chapter four** explored the role of PduCDE enzyme in the ecological performance of *L. reuteri*. From a series of *in vitro* cross-feeding experiments it was concluded that *L. reuteri* can cross-feed from 1,2 PD-produced by strains of *Escherichia coli* and *Bifidobacterium breve* from the fermentation of deoxyhexoses found in the gut. During fermentation of hexoses, 1,2 PD serves as an electron acceptor increasing the metabolic efficiency of *L. reuteri,* a factor that could be pivotal to the competitiveness of *L. reuteri* in the gut ecosystems. These findings are exciting because L-rhamnose, a precursor of 1,2 PD, can be introduced into the human gut via dietary plant-derived polysaccharides. Based on these results, our group is currently planning dietary intervention trials aimed to determine if increasing

the consumption of rhamnose-containing foods, and thus the availability of 1,2 PD in the gut, can support the persistence of *L. reuteri* in humans.

Finally, work in **Chapter five** (Duar *et al.* 2015) explored an innovative strategy to select and exploit the metabolic capacity of lactobacilli adapted to the vertebrate gut. Strains of *L. salivarius, L. amylovorus. L ruminis* and *L. johnsonii* isolated from the pig's gastrointestinal tract demonstrated the ability to degrade metastable peptides implicated in the pathology of celiac disease. Although these strains are not of human origin, they are likely to be active the conditions of the proximal gastrointestinal tract. This characterictic could be advantageous as gluten peptides would need to be degraded before reaching the intestinal epithelium. Furthermore, findings in **Chapter 2** demonstrated that porcine strains of *L. reuteri* are competitive in the human gut. The gastrointestinal tract of pigs are anatomically and physiologically similar (Heinritz, Mosenthin and Weiss 2013). However, contrary to pigs, humans harbor relatively low amounts of lactobacilli. Therefore, sourcing strains from the porcine hosts could pose a suitable strategy to select for strains with probiotic potential in humans. Together these chapters provide a basis for the selection of lactobacilli strains aimed to be competitive and physiologically active in the human gastrointestinal tract.

6.2 Implications

The implications of findings from this dissertation extend beyond its contribution to a basic understanding of the biology and ecology of the genus *Lactobacillus*. Humans have essentially "domesticated" lactobacilli for use in food and feed production, an increased understanding of the origin of these microbes and their function in nature will therefore facilitate the selection of strains for such applications. Attributes that lactobacilli that evolved in their natural habitats, such as metabolic functions, antagonism towards other members of microbial communities, and their impact on host species, can be exploited once understood. In addition, an understanding of host-associated lactobacilli might allow the development of strategies to support their populations or

beneficial metabolic activities through dietary intervention. For example, strong attention has been paid towards the unsolved decline in the population of honey bees (Goulson *et al*. 2015; Engel *et al*. 2016). Their paramount importance as pollinators of agricultural crops justifies efforts to understand and better manage their symbiotic interactions with microbes as a tool to preserve insect health (Engel *et al.* 2016). Similar considerations also apply to humans and farm animals, many of which maintain dominant population of lactobacilli among their microbiota (i.e. swine and poultry). The framework presented in **Chapters one, two** and **five** lay a foundation of strategies to select strains for a whole range of biotechnological and therapeutic applications.

Furthermore, as demonstrated in **Chapter two** and by others (Oh *et al*. 2010; Frese *et al.* 2011), host-adapted strains of lactobacilli show a higher ecological fitness in their respective hosts Therefore, host-specific lactobacilli are likely to be more competitive when administered as a probiotics, compared to strains that do not share an evolutionary history with the host. Higher fitness is relevant for the development of probiotics supposed to outcompete pathogens, and it is likely to be associated with higher metabolic activity in the host niche, which could lead to an increased production of metabolic compounds that define probiotic activity. In addition, stable transmission of bacterial symbionts over evolutionary times promotes traits that enhance partner performance (Herre et al. 1999; Sachs et al. 2004; Douglas 2008). Providing this theory holds true for the relationship between lactobacilli and animal hosts, then host-adapted *Lactobacillus* strains that share an evolutionary fate with their host are more likely to possess adaptive traits that enhance health of their host.

Such evolutionary aspects have rarely been considered for the selection of strains for specific applications. It is a logical working hypothesis that host-adapted *Lactobacillu*s strains will show higher levels of ecological performance when used as probiotics, possess beneficial traits that enhance host fitness, and are likely to establish interactions with the host immune system that are characterized by tolerance (Walter, Britton and Roos 2011). Conversely, if the aim is to stimulate the immune system, selection of species or strains that lack a joint evolution with a host

may be a more sensible approach. This proved true for *L. reuteri* in which strains from the autochthonous human lineage had an anti-inflammatory effect in human myeloid cells while strains associated with the phylogenetic lineage that evolved with poultry had a rather stimulatory immune effect (Spinler *et al*. 2014). These findings highlight the functional significance of the natural history of lactobacilli for probiotic functions. Although one cannot generalize what constitutes a better probiotic - host-adapted or not – the evolutionary history of a strain will fundamentally influence its functionality. A consideration of the natural history of lactobacilli will therefore aid in the more systematic and targeted selection of optimal strains for specific therapeutic applications. The framework established in **Chapters one** and **two** can clearly provide guidance for such a selection.

Finally, if as hypothesized, findings in **Chapter two** regarding the inability of human strains of *L. reuteri* to persist in the human gut, are explained by findings in **Chapter four**, then increasing rhamnose in the diet could represents a suitable strategy to increase production of 1,2 PD in order to support the persistence of *L. reuteri* in humans. Dietary interventions aimed to increase rhamose in the diet are therefore warranted to shed light into the relevance of 1,2 PD *in vivo* and would provide a basis for dietary strategies aimed to support other human gut symbionts.

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