Impact of probiotics on commensal and pathogenic swine intestinal microbes

by

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Abstract

The application of probiotics is considered an effective approach to improve overall health conditions and growth performance in swine. Understanding the properties of these probiotics is a prerequisite for the selection of probiotics for pigs. Here, a rational selection criteria of swine probiotics is discussed mainly based on divergent probiotic effects of different strains and practical purposes in swine production (Chapter 2). The systematically investigated ecological history rather than the source of isolates should be regarded as the natural origin of probiotic strains, which helps to correct the inconsistencies arising from incorrect identification of the source. Host-adapted probiotic strains are likely to associated with exclusive colonization while the nomadic or environmental strain exert better immune stimulating functions. Strains with potent enzymatic activity are fitter for grower pigs favoring feed digestion and enhancing growth performance.

Enterotoxigenic *Escherichia coli* (ETEC) is known to be a major pathogen in swine, however, this group of pathogens has a variable profile. Monitoring this profile of ETEC virulence factors could supply novel strategies to develop ETEC vaccines or fimbriae receptor analogues. In this thesis, a multiplex high-resolution melting curves-based qPCR (HRM-qPCR) assay was developed which enabled quantification of predominant ETEC fimbriae types and additionally monitored the presence or absence of other fimbriae types related to post-weaning diarrhea. Inclusion of host-adapted *L. reuteri* effectively reduced ETEC abundance in swine intestine (Chapter 4 and 5). In contrast, nomadic *L. fermentum* and *L. casei* did not show inhibitory effects on ETEC but eliminated *Clostridium* spp.. Furthermore, probiotic intervention showed limited alteration of commensal gut microbiota.

The feed transition after weaning also induces an increasing ability of intestinal microorganisms to harvest energy from dietary carbohydrates. To unravel the adaptation of the swine microbiome

to the dietary shift after weaning, a total of 360 high-quality microbial genomes were assembled as the first metagenomic-assembled-genomic reference for swine intestinal microbiota (Chapter 6). The reconstructed gut microbiome allowed identification of key microbial contributors to the degradation of starch, fructans, and lactose. Starch is a substrate for colonic microbiota and its metabolism is dependent on metabolic cooperativity between *Firmicutes* and *Bacteroidetes*. The functional prediction of the most abundant extracellular starch degrading enzyme in *Firmicutes* was further validated by protein purification and activity assays (Chapter 7). Fructans and lactose are fermented by simple enzymatic systems present in *Bacteroides* and *Lactobacillus* spp., respectively. The improved understanding of carbohydrate fermentation in the swine intestine enables the development of feeding models with higher feed efficiency and better pathogen control for weanling pigs.

Preface

This thesis is an original work by Weilan Wang.

Chapter 2 has been published as: Wang WL and Gänzle MG. "Towards rational selection criteria for selection of probiotics in pigs" *Advances in applied microbiology*. I assembled the relevant information and wrote the manuscript with guidance from Dr. Gänzle.

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Chapter 6 has been published as: Wang W, Hu H, Zijlstra RT, Zheng JS, Gänzle MG. "Metagenomic reconstructions of gut microbial metabolism in weanling pigs". *Microbiome.* Drs. Gänzle, Zheng and Zijlstra designed the experiment. I conducted animal study, analyzed bioinformatic data with Huifeng Hu, wrote the manuscript with Dr. Gänzle.

Chapter 7 contains experimental works performed by me with guidance from Dr. Gänzle.

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List of Abbreviations

AFLP	Amplified fragment length polymorphism
ANI	Average nucleotide identity
ANOSIM	Analysis of similarities
CDI	*C. difficile* Infection
CEs	Carbohydrate esterases
COG	Clusters of Orthologous Groups
CPB	Necrotizing β-toxin
C-section delivery	Caesarean section delivery
DC	Dendritic cells
DM	Dry matter
ETEC	Enterotoxigenic *Escherichia coli*
GHs	Glycoside hydrolases
GI tract	Gastrointestinal tract
GlgB	α-(1→4)-glucan branching enzyme
HMOs	Human-milk oligosaccharides
HRM-qPCR	High-resolution melting curves-based qPCR
HTC	Hybrid two-component
IL	Interleukin
LB	Luria-Bertani
LME	Linear mixed-effects
LT	Heat-labile toxin
ORFs	Open reading frames

OTU	Operational Taxonomic Units
PCoA	Principle Coordinate Analysis
PFGE	Pulsed-Field Gel Electrophoresis
PIS	Porcine intestinal spirochaetosis
PMOs	Porcine milk oligosaccharides
PPE	Porcine proliferative enteropathy
PWD	Post weaning diarrhea
RAPD	Random Amplification of Polymorphic DNA
RDA	Representational difference analysis
SD	Swine dysentery
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSH	Suppression Subtractive Hybridization
STa	Heat-stable toxin a
STb	Heat-stable toxin b
STEC	Shiga-toxin producing *E. coli*
T reg	Regulatory T cells
Th	T helper cell
TLR	Toll-like-receptor
Tm	Melting temperature

Chapter 1. Introduction

1.1. Application of antibiotics and probiotics in swine production

The primary aim of swine production is to maintain overall health and to improve the growth performance of pigs. Antibiotics, including prophylactic antibiotics and growth promoting antibiotics [1] are one of the most cost-effective choices to maintain gut health and promote animal growth [2]. Direct inhibition on gut microbiota is regarded as the major mechanism of antibiotics promoting growth. The mechanism relying on suppressing the competitiveness of gut microbiota for nutrients contributes to increased feed efficiency in animals [3]. In addition to providing medication, antibiotics have been used at a subtherapeutic level to protect animals from infection by opportunistic pathogens [4,5]. However, the growing spectrum of antibiotic resistance in human and animal pathogens raises the concern of antibiotic abuse and has led to the banning or restriction of antibiotics as growth promoters in many jurisdictions.

Research on alternatives to antibiotics included studies on organic acids, probiotics, prebiotics and minerals [6] to reduce the dependence of antibiotics in animal production. Probiotics have been considered as a promising option due to their health promoting effects in humans and animals. The earliest study of swine probiotic was reported in the 1970s. *L. delbrueckii* fermented broth showed anti-*E.coli* activity in early weaned pigs [7]. Subsequent studies also showed beneficial effects of *Lactobacillus, Bacillus, Enterococcus* and *Pediococcus* in pigs [8–11]. However, inconsistent results have been reported in studies supplying various strains through different delivery methods to pigs [12–15]. These inconsistencies call for more credible analysis and systematic elucidation to correct the differences caused by misdescription on the properties of probiotic strains and their practical effects [16,17]. In addition, the variable beneficial traits required by pig at different

growth stages [18,19] should be carefully distinguished for probiotics selection in large scale production.

1.2. Hypotheses and objectives

Hypotheses

- Probiotics reduce risk of infection by intestinal pathogens in current swine production systems, but their effect is dependent on the ecological adaptation and the corresponding metabolic traits of the probiotic strain (Chapter 2, 4 and 5).
- Swine gut microbiome adapts to the change in the diet after weaning (Chapter 6).
- GlgB branching enzyme catalyzes the formation of α- (1→6)-branches on amylose (Chapter 7)

Objectives

- Selection criteria of probiotics for pigs differ at different growing stage (Chapter 2)
- Improve high-resolution melting curve-based methods for quantitative detection of swine related ETEC fimbriae (Chapter 3)
- Evaluate impact of various lactobacilli on commensal microbiota and pathogens (Chapter 4)
- Lactobacilli with different ecological origins have divergent probiotic effects of (Chapter 5)
- Unravel the adaptation of the swine microbiome to the dietary shift after weaning by metagenomic reconstruction and biochemical characterization of key enzymes involving in major carbohydrates metabolism (Chapter 6 and 7).

Chapter 2. Towards rational selection criteria for selection of probiotics in pigs

2.1. Introduction

The number of microorganisms with claimed probiotic effects or that have been developed into commercial products has boomed in recent decades, however, results in practice have led to uncertainty about their beneficial roles. Probiotic have been primarily selected in vitro for high acid and bile salt resistance, viability during drying and feed processing, certain enzymatic activities, and anti-microbial activity [12–15]. While these traits may impact probiotic activity, the reliability of in vitro evaluation criteria has been questioned [20]. Moreover, discrepancies between in vivo evaluation and efficacy in commercial practice indicate that knowledge on selection criteria for probiotics is incomplete.

In animal production, probiotics have been applied as feed additive for growth promotion and inhibiting pathogenic bacteria for >30 years [21,22]. Highly diverse organisms of the genera *Lactobacillus*, *Bacillus*, *Enterococcus* and *Pediococcus* are most commonly applied in swine production [8–11]. The physiological and microbiome development of pigs after birth [18,19,23], in combination with the different animal production systems [24,25] may require a different function of probiotics to achieve different health beneficial effects. However, few of the past contributions consider the necessity to select for different probiotics at different stages of the animal's life. Therefore, this review aims to assess selection criteria for selection of probiotics in pigs. The assessment is based on a short description of the development of the intestinal microbiome, and the major pathogens in pigs.

2.2. Initial acquisition of early neonatal microbiome

The initial acquisitions of gut microbes in mammals is best characterized in humans. In neonates, gut microbes guide the development of the gastrointestinal tract (GI tract) and educate the immune system [26,27]. At birth, the neonatal gut is aerobic and is initially colonized by facultative aerobes and aerobes [28]. These facultative aerobes, including *Escherichia* and *Enterococcus* are pioneering colonizers and mediate the shift to an anaerobic condition in the first postpartum week [26,28]. Maternal microbial transmission and the exposure to environment shape the early microbiota composition of newborns [28–30].

2.2.1. Sow-to-piglets transmission sets the stage for the gut microbiome

Comparable to human neonates, the vertical transmission of bacteria from sow to piglets is influenced by the mode of delivery [27,31], the feeding method and the contact with the sow [28,29,32]. Animal studies associated caesarean section (C-section) delivery with delayed gut maturation and microbial colonization [31], reduced growth rate, and altered immune system function in piglets [33]. The phylogenetic patterns were also distinct between vaginally delivered and C-section delivered infants, especially the abundance of typical vaginal lactobacilli [28,34]. The dramatical reduction of these lactobacilli within the first postnatal week in human suggested that vaginal microbes are more likely transient rather than residents of neonatal hindgut [27,28,34]. Lactobacilli are not dominant members of swine vaginal microbiota [35] but members of sow vaginal microbiota may also temporarily colonize the piglet's intestine.

Gut microbiome and mother milk are the two validated maternal sources of vertical microbial transmission [28,30,36]. After the initial acquisition of the microbiome at birth, piglets are continuously exposed to niche-adapted microbes transmitted through the intimate contact with other animals [37]. Maternal transmitted strains showed better fitness for colonization than strains

acquired later from non-maternal sources, suggesting that first colonizers can defend ecological niche organisms that arrive later to compete for the same niche [28,30,36]. Continuously exposure to non-maternal microorganisms, however, leads to the decrease of the number of shared bacterial species between mother and infant over time [28]. In human, the vertical transmission of bifidobacteria is supported by human-milk oligosaccharides (HMOs), which selectively stimulate bifidobacteria in the infant's gut [28,30,38,39]. Porcine colostrum contains around 12g/L porcine milk oligosaccharides (PMOs); the concentration of PMO decreases to 7g/L in the mature milk [40–42]. The absence of intact PMOs at the first day of farrowing [42] and the presence of the same strains of lactobacilli in sow milk and piglets suggested the possibility of vertical transmission of oligosaccharide degraders through sow milk [15,36]. Sow milk is also the primary carrier of nutrients and immune regulatory components to feed and protect piglets [26,43]. All these studies suggest that early life of piglets provides a "window of opportunity" to educate immune system and microbial communities in neonates by applying probiotic therapy in sows.

2.2.2. Continuous host cross-talk with microbes shapes the developing gut microbiome

At birth, the newborn is readily colonized by immigrant microorganisms from maternal and non-maternal sources, resulting an overall high diversity and heterogeneity of colonizers in the first few days of life [26,28–30]. Subsequent niche-specific selection, however, shapes gut microbiota into a stable ecology that is dominated by representatives of five bacterial phyla [17,28,30]. The higher competitiveness of co-evolved symbionts for adhesion sites and resources allows their use in microbial-based strategies to modulate gut microbiomes, which has been particularly well described in lactobacilli. [17,44,45]. More remarkably, the earlier colonizers exert high selective pressure on the subsequent arrivers. The sequence of arrival of these residents can be random but significantly impacts the individual composition of the microbiome [46]. This contingency of

colonization history of gut microbiota in newborn has been considered as a potential explanation for the high levels of unexplained individual variation [46]. Host-adapted strains are more likely to overcome the habitat filters and to outcompete other residents, especially, especially when they are applied as probiotics in early life [16,47].

However, the homeostasis of microbiota is far from impregnable but continuously interrupted by multiple factors. Weaning is regarded as a critical phase of pig's early life, in current swine productions, animals are generally around 3-4 weeks post-partum. This artificial intervention abruptly interrupts immune protections from mother milk, imposes social stress by separation from mother and littermates, and forces the young animals to adapt to less-digestible solid feed [48]. These social and physical stresses correspond to severe dysbiosis of gut microbiota and a high susceptibility to pathogen infection in weaning pigs [49,50]. The dietary shift and physiological development alone induce a major succession of intestinal microbiome [18,23,51]. One of the most common reported microbial succession is *Lactobacillus spp.* due to the milk interruption at weaning [23]. Longitudinal studies of intestinal microbiome of weaning piglets revealed a dramatical decrease of *L. delbrueckii* after weaning, an organism which maintains a very selective metabolic focus on lactose ([23]. A second example is the increase of *Bacteroidetes* in response to the inclusion of plant-derived polysaccharides in feed after weaning [18,23,52]. The decline of the abundance of *Enterobacteriaceae* in the late phase of weaning suggested a progressively increasing resistance of weaned piglets to pathogen infection [18,23,51]. These changes of gut microbes stabilize typically within 2-3 weeks after weaning [18,23,52].

2.3. Bacterial-induced enteric disease in pigs

The gastrointestinal tract is a reservoir of various pathogens which induce different enteric diseases and cause severe economic loss in swine industry. These pathogens infect pigs by a variety of

mechanism and generally exhibit dynamic patterns of prevalence at various ages of pigs (Table 2.1). The higher susceptibility to bacterial infections in young animals relate to the immature immune systems, the developing gastrointestinal barrier and to microbial dysbiosis induced by weaning transition [53–55]. Grower / finisher pigs are less susceptible to enteric infectious, but the disease related morbidity and growth retardation are more costly, and are associated with a higher cost of in-feed medication [56–58]. Disease in later stage of life, however, can be controlled by vaccines while probiotics can be an alternative way for suckling and weanling piglets.

Table 2.1. Frequency of major porcine enteric diseases at different growth stages

Infected agent	Clinic disease	Newborn	Suckling (0-3 weeks)	Weaning (4-10 weeks)	Grower (11-14 weeks)	Finisher (>15 weeks)	Reference
ETEC K88	Post weaning diarrhea (PWD)	+++	++++	++++	−	−	[59–63]
ETEC F18	PWD	−	−	++++	−	−	[59–63]
C. difficile	*C. difficile* Infection (CDI)	++++	+++	−	−	−	[64–66]
C. perfringens Type C	Type C infection	++++	+++	−	−	+	[57,63,67,68]
C. perfringens Type A	Type A infection	+	+	++	+++	++++	[67,68]
S. enterica	Salmonellosis	+	−	++	+++	++++	[57,58,63,69–71]
B. hyodysenteriae	Swine dysentery (SD)	−	−	+++	+++	++++	[56,57,72,73]
B. pilosicoli	Porcine intestinal spirochaetosis (PIS)	−	−	+	++++	++	[56,57,72]
L. intracellularis	Porcine proliferative enteropathy (PPE)	−	+	+++	++++	++++	[63,72,74,75]

Note: '-' represents pigs are seldom infected; More '+' represents increasing frequencies of infection. The division of the pig stages is based on the code of practice for the care and handling of pigs in Canada (Canada, 2014).

2.3.1. ETEC and Clostridial infection in early life of pigs

Enterotoxigenic *Escherichia coli* (ETEC) is one of the most commonly bacterial pathogens in neonatal and post-weaning pigs [54]. ETEC bacteria adhere to small intestinal microvilli by host-specific fimbriae, and then secrete enterotoxins which cause severe diarrhea by perturbing the secretion and absorption of water by enterocytes [76]. In swine, five swine-specific fimbriae, K99, F41, F18, F6 and K88, mediate adhesion to the swine intestinal mucosa. Of these, organisms expressing K88 and F18 cause a majority of diarrheal diseases in pigs [77,78]. K88 and K99 fimbriae positive ETEC are more frequent isolated in neonate piglets while ETEC carrying F18 fimbriae are mostly related to edema disease or post-weaning diarrhea. This age-specific pattern was related to the absence of the oligosaccharide receptor that mediates binding of F18 fimbriae in newborns [78–80]. The pattern of virulence factors also exhibits specific combinations. ETEC strains expressing K88 fimbriae more frequently produce heat-labile toxin (LT) and heat-stable toxin b (STb) while F18 positive strains more frequently produce heat-stable enterotoxins (STa and STb) [81,82]. ETEC is a non-invasive pathogen, therefore, the mucosal F4- and /or F18-specific secretory IgA is more effective to prevent ETEC colonization than the lactogenic immunity stimulated by maternal vaccines which decreases with aging and is interrupted by weaning [77,83]. Moreover, limited success was reported in oral administration of vaccines with or without encapsulation in suckling and weaned pigs [84,85]. Thus, additional measures are required to protect neonates and weanling pigs against ETEC infections.

Clostridoides difficile and *C. perfringens* Type C are the main infectious agents of clostridial enteric infections in neonatal pigs at 1 to 7 days of age [68]. In swine production, the prevalence of *C. difficile* infection can be as high as 50% to nearly 100% in piglets between 0- 2 weeks of age but this high prevalence gradual declines as piglets grow older [68,86,87]. Most toxigenic *C.*

difficile isolates from pig produce enterotoxin tcdA and cytotoxic tcdB, which induce exudative colitis and epithelial cell collapse and death, respectively [65,87]. *C. difficile* spread easily between sows and piglets through the environment as well as vertical transmission. Accordingly, *C. difficile* was isolated in neonatal piglets soon after birth but no culture was obtained from C-section delivered piglets [88]. *C. perfingens* Type C infection is characterized by high incidence of hemorrhagic necrotic enteritis in piglets < 7 days of age [68]. The key factor mediating type C infections is the lethal and necrotizing β-toxin (CPB) [67,89,90]. Prevalence of *C. perfringens* type C can be 100% in infected litters, with mortality rate as high as 50-60% [57,67,91]. Disease caused by *C. perfringens* type C has been well controlled by vaccination in swine production in recent years [90].

2.3.2. Prevalence of bacterial induced colitis in later life of pigs

Brachyspira hyodysenteriae, B. pilosicoli and *Lawsonia intracellularis* are the main infectious agents that cause swine dysentery (SD), porcine intestinal spirochetosis (PIS) and porcine proliferative enteropathy (PPE) in grower / finisher pigs, respectively. Although the precise pathogenesis of SD is still unclear, the infected pigs present reduced feed intake, a depression of growth rate and soft or porridge-like diarrhea [63,73,92]. PIS caused by *B. pilosicoli* is distinct from *B. hyodysenteriae* and normally does not lead to the death of pigs but results in reduced growth rate and increased time to market weight [56,57,93]. PPE infection is commonly found in pigs aged from 6 to 20 weeks. PPE induces acute disease in older pigs and chronic conditions in young growing pigs [75,94]. The acute form is observed with hemorrhagic diarrhea and sudden death while the chronic form is observed with signs of diarrhea, rough hair-coat and retarded growth [95,96]. *Salmonella* Typhimurium is the serotype that is most commonly associated with salmonellosis in piglets and growing pigs although a wide range of serotypes were also isolated in

sow [71]. *Salmonella* generally infects the ceca and induces loss of appetite, a febrile response with dullness and watery diarrhea in growing pigs [97,98]. The long persistence of strains in various tissue in growing-finishing pigs also concerns food safety through contamination of pork [63,99].

2.4. Probiotic at different stages of life

In modern swine production, especially intensive industry, different management practices respond to the diverse physiological characteristics of pigs at different growth stages to maximize production efficiency. Accordingly, desired properties of probiotic strains and the major purposes of applying probiotic also vary with the growth stage.

2.4.1. Application of probiotics in sow/neonates

Inclusion of probiotic *Enterococcus faecium* NCIMB 10415 in feed supplementation is a success case which exerted positive effects both on sow and newborns that were consistently demonstrated in multiple studies [37,43,100,101] (Table 2.2). Supplementation of sow feed with *E. faecium* NCIMB 10415 reduced the weight loss of sow and increased the content of lactose as well as the percentage of milk cells expressing mCD14 in sow milk, which positively correlated to weaning weight and the percentages of B cells and activated T cells in piglets [11,102,103]. *E. faecium* does not permanently colonize the intestine [104,105], therefore, feeding sows and their piglets with the same strain simultaneously was more effective than feeding sows alone [11,102,106]. Continuously supplying *E. faecium* NCIMB 10415 to suckling pigs improved their growth performance and decreased the incidence of diarrhea [43,107,108]. Similar results were observed in animals studies applying *Bacillus*-based probiotic to sow and piglets [9,109,110] (Table2.2). These findings suggested that simultaneously introducing probiotic in feed for sow

Table 2.2. Application and observations of probiotics in pigs

Application	Strain	Source and ecotype	Dose (CFU/d)	Duration (d)	Positive observations					References
					Improved growth performance	Regulated gut microbiome	Anti-inflammation / Immunomodulation	Reduced pathogen load	Improved reproductive performance	
Neonatal piglets	*B. subtilis* C3102	− / environment	10^9	26	+	−	−	+	N/A	[109]
	B. licheniformis / *B. subtilis*	soil / environment	10^9	40	+	−	−	+	N/A	[9]
	L. fermentum I5007	Pig / nomadic	10^{10}	14	−	+	+	+	N/A	[111]
	E. faecium NCIMB10415	−	10^9	54	−	+	+	+	N/A	[100,101]
	E. faecium EF1	−	10^9	25	−	−	+	−	N/A	[112]
	L. salivarius B1	pig (M)	10^9	28	−	−	+	−	N/A	[10]
Weaned piglets	*L. fermentum* I5007	pig (M)	10^9	10	−	−	+	−	N/A	[113]
	L. brevis ATCC 8287	fermented green olives	10^{10}	21	−	−	+	−	N/A	[114]
	L. rhamnosus GG	human	10^{10}	14	−	+	+	+	N/A	[115]
	E. faecium CECT 4515	−	10^9	56	+	+	−	−	N/A	[116]
	L. reuteri and *L. plantarum*	−	10^9	28	+	−	−	+	N/A	[117]
	L. reuteri TMW1.656	sourdough	10^{10}	21	−	+	−	+	N/A	[118]
Growing / Finishing pigs	*E. faecium* NCIMB10415	−	10^{10}	56	+	−	−	−	N/A	[119]
	B. lichenformis / *B. subtilis*	−	10^{11}	60	+	−	−	−	N/A	[120]
	L. plantarum ZJ316	human (infant)	10^9	60	+	−	−	−	N/A	[121]
	L. acidophilus NCDC15	dairy products	10^{11}	180	+	−	−	−	N/A	[8]
	P. acidilactici strain FT28).	pig	10^{11}	180	+	−	−	+	N/A	[8]
Sow	*E. faecium* DSM 7134	−	10^8	58	+	−	−	−	−	[11]
	B. licheniformis / *B. subtilis*	soil / soya bean fermentation	10^9	40	+	−	−	−	+	[9]
	B. subtilis C3102	−	10^9	130	+	−	−	−	+	[109]
	L. johnsonii XS4	pig (M)	10^9	75	+	−	−	−	+	[122]
	E. faecium NCIMB10415	−	10^9	56	−	+	−	−	+	[37,43,101]
	B. cereus var. *toyoi* NCIMB 40112	−	10^9	120	−	−	+	−	−	[123]

and suckling pigs could enhance the overall benefits effects both on sow and piglets more efficiently.

2.4.2. Application of probiotics in weanling piglets

The purpose of applying probiotics in weanling pigs aims to alleviate the weaning stress or to restore gut microbiota from weaning dysbiosis. Because the control of non-invasive pathogens infection by vaccination is limited [83], the ability to inhibit pathogen colonization has been considered as the primary aim of probiotics application in weanling piglets. Probiotic effects against pathogens were shown in pathogen challenged animals [124–126] (Table 2.3). *L. salivarius, L. reuteri* and *L. amylovorus* increased growth performance of weanling pigs after challenge with pathogenic *E.coli* or *Salmonella* and reduced pathogen counts without significant immunomodulation [124,126,127]. In contrast, *L. rhamnosus*, *L. plantarum* and *Bacillus* cleared pathogens by stimulating host immune response [113,124,125,128–130]. These two distinct modes of action correspond to different ecological roles of host-adapted and normadic lactobacilli [44,131]. *L. salivarius, L. reuteri* and *L. amylovorus* are host-adapted strain which share a long-term evolutionary history with swine while *L. rhamnosus*, *L. plantarum* and *Bacillus* are nomadic or environmental strains which is able to persist in different habitats [44,131].

Molecular mechanisms involving in divergent immune responses induced by autochthons and allochthons are well studied in human and mice (Figure 2.1)[132–134]. Host-adapted *B. infantis*, *B. breve* and *L. reuteri* skewed immune response toward tolerance by suppression proinflammatory cytokines in dendritic cells (DC) and induced Foxp3+ regulatory T cells (T reg) producing anti-inflammatory cytokine IL-10 [132,134–136]. In contrast, higher level of proinflammatory cytokines were elicited in DCs by allochthonous *L. rhamnosus* and *L. plantarum*, thus inducing an inflammatory response [132,134] (Figure 2.1). Although *in vivo* data for swine

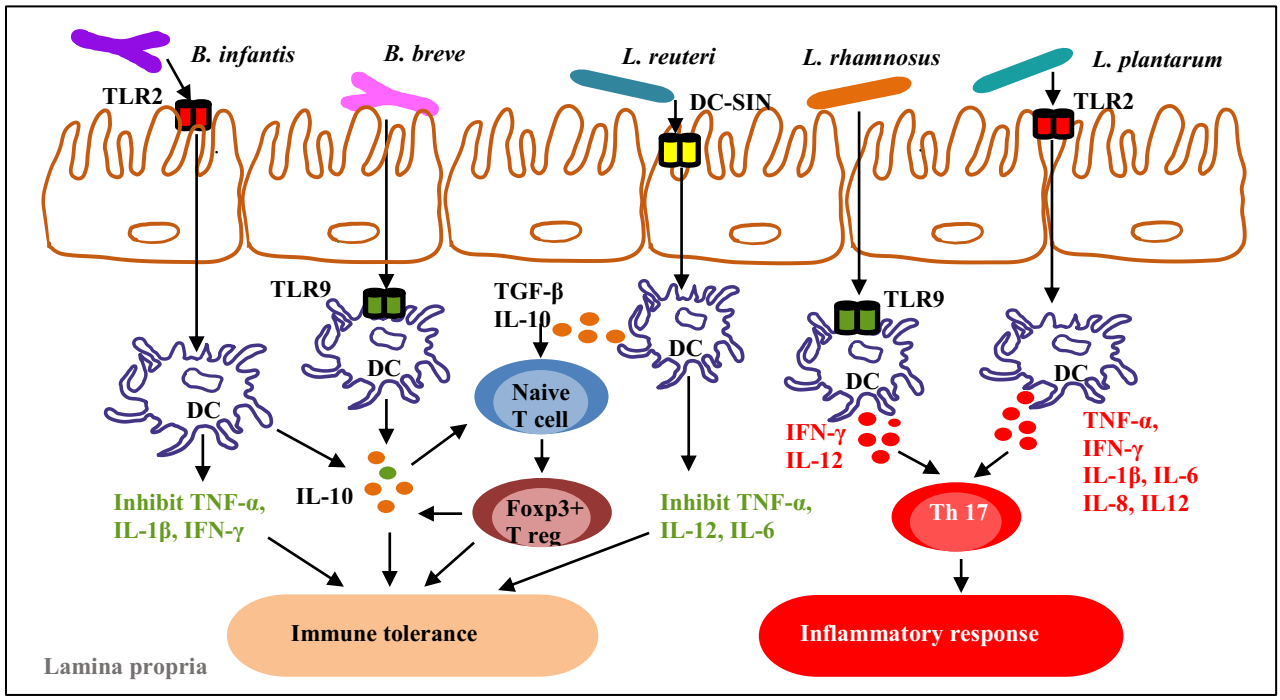
that would ascertain translation of mechanism from human or mice to pigs are currently insufficient, it is nevertheless credible that host- adapted probiotic strains differ from nomadic strains with respect to their interaction with the immune system. In vitro data support the notion that host adapted strains are associated with immune tolerance while the nomadic or environmental strain are immune stimulatory.

Applications of different doses of the same strain as probiotic in weaning pigs suggested the dose plays an important role for probiotic effects,, but more is not always better [125,137]. Generally, continued feeding of probiotic organisms is required to maintain relevant cell counts of probiotics in the intestine [138,139].

Table 2.3. Application and observations of probiotics in pathogen challenged piglets

Pathogen (CFU /d)	Probiotic (CFU /d)	Source and eco-type	Observations			Reference
			Immunomodulation	Pathogen load	Growth performance	
10^9 S. *enterica serovar* Typhimurium +*E. coli*	10^{10} *B. subtilis* and *B. licheniformis*	- / environment	elevated serum IgG level	reduced fecal pathogen counts	-	[124]
10^{11} *Salmonella* Typhimurium	10^{10} *B. subtilis* and *B. methylotrophicus*	- / environment	increased level of RBC, IgG, and IgM	reduced Salmonella counts	-	[128]
10^9 *Salmonella* Typhimurium	10^8 *B. cereus var*. Toyoi	- / environment	reduce CD8+ T cells in the peripheral blood and jejunal epithelium	reduced fecal shedding of Salmonella	-	[129]
10^{10} ETEC K88	10^{10} *L. rhamnosus*	Human / nomadic	expressed jejunal TLR2, ileal TLR9; upregulated of Nod-like receptor NOD1 and TNF-a mRNA	-	-	[125]
10^9 ETEC K88ac	10^9 *L. plantarum*	- /nomadic	increased blood CD4+ lymphocyte subset percentage TNF-α and interferon-γ in the ileum	-	-	[113]
10^{10} ETEC K88ac	10^8 *B. licheniformis* and *B. subtilis*	- / environment	excessive generation of CD4+IL-10+ T cells	-	-	[130]
10^8 ETEC K88	10^9 *L. salivarius*	- / vertebrate-adapted	-	-	improved villous height and growth performance	[126]
10^{10} ETEC K88ac	10^{10} *L. amylovorus*	- / vertebrate-adapted	-	reduced ETEC content in ileum	Increased ADG	[127]
10^{10} *S. enterica serovar* Typhimurium +*E. coli*	10^{10} *L. reuteri*	- / vertebrate-adapted	-	reduced fecal pathogens counts	Increased ADG	[124]

Figure 2.1. Different interactions of bifidobacteria and lactobacilli with immune cells in mucosa. Lower inflammatory response was induced by host-adapted bifidobacteria and *Lactobacillus reuteri* than allochthons lactobacilli. *B. infantis* suppressed the secretion of proinflammatory cytokines TNF- α, interleukin (IL)-1β and IFN-γ in dendritic cells (DC) via Toll-like-receptor (TLR)-2 signaling and induced Foxp3 regulatory T cells (T reg) releasing high level of IL-10 [132]. *B. breve* activated Foxp3+ T reg producing high level of IL-10 in a TLR9-dependent manner [132]. *L. reuteri* down-regulated proinflammatory cytokines TNF- α, IL-12 and IL-6 through DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and promoted Foxp3+ T reg secreting high level of IL-10 and TGF- β [134,136]. *L. rhamnosus* and *L. plantrarum* facilitated inflammatory response by upregulating cytokines (IFN-γ, IL-12, TNF- α, IL-1β, IL-6 and IL-8) and elicited T helper cell (Th)-17 through TLR9 and TLR2 signaling, respectively [132].



2.4.3. Application of probiotics in grower / finisher pigs

During growing-finishing stage, bacterial infection is controllable through vaccination [140–142], and the primary goal becomes to improve growth performance. Correspondingly, the required characteristics of probiotic strains shift towards favoring digestion and providing metabolic energy. *Bacillus subtilis* is a potent producer of hydrolytic enzymes including cellulases and phytases [12], which contributes to the improved growth performance of the grower pigs feed with *Bacillus*-based probiotics [120,143–145]. Similarly, *L. plantarum* was found to improve pig growth primarily benefiting from its outstanding metabolic capacity during feed fermentation [121,146]. Although both *L. acidophilus* NCDC-15 and *P. acidilactici* FT28 showed positive effects on growth performance of grower-finisher pigs, the latter strain was more effective due to the higher metabolic capacity [8]. Another important common thread in these success cases is supplying probiotics with a high dose (> 10^{10} CFU /d) and for a long period (> 60 days) [8,119,121]. By contrast, limited improvemets was oberved when *Bacillus* was provided only for a short time[147].

2.5. Selection criteria for probiotic application

To achieve diverse purposes of applying probiotics in swine production, different beneficial profiles are required for probiotic strains. An overview on the relevance of different selection criteria at different stages of life is provided in Figure 2. 2. Only pig-adapted strains that are administrated in early life or shortly after antibiotic treatment are likely to colonize in intestine of piglets [16,17,20,44,46,148]. Host adapted lactobacilli are tolerant to acid and bile salt [149], and possess the metabolic characteristics [12] to compete for adhesion sites and nutrients in swine gut [16,148]. Altering the type of early intestinal colonizers in mice showed a lasting influence on the development of microbiome [46], indicating the possibility to shape the development of gut

microbiota of neonates by introducing host-adapted probiotics early in life. In addition, higher competitiveness of these strains to outcompete pathogens is of importance for piglets who cannot receive full protection from the immature immune system. Thus, to educate the development of gut microbiota or to competitive exclude pathogen attachments, it is necessary to select pig-adapted probiotics. Both phylogenetic inferences and associated functional studies should be combined to elucidate the host adaption of certain strains or species, rather than simply depending on the sources of isolation [16,148]. To date, studies on host-adaption have been limited to *Lactobacillus;* a systematical investigation of host adaption in other species used as probiotic remains subject to future investigation.

Probiotics also protect piglets by generating antimicrobila compounds or upregulating immunity against pathogenic infection [12,14,15,149–152]. The production of organic acids, exopolysaccharides and other antimicrobial compounds is independent from the lifestyle of probiotics and was shown to contribute to pathogen inhibition in weanling pigs [118,153,154]. Compared to host-adapted probiotics, nomadic or environmental probiotics are more likely to clear pathogens by stimulating immunologic defenses [113,124,128,129]. Accordingly, *in vitro* identification of immunomodulatory traits of a strain has been widely used to predict its potential use as probiotic, which also has raised a concern about the validity of these *in vitro* studies. Most of positive results detected in IPEC-J2 cell based evaluation were not validated by animal studies or the results from animal studies were not as significant as predicted by cell line test using the same strain [104,105,117,121,155–158]. In terms of inhibiting pathogen infections without colonization, both host-adapted and non-host-adapted probiotics can provide effective protections either through sow milk or directly feeding suckling and weanling pigs [11,102,103,113,124,125,128–130]. But the precondition is selecting probiotics with high

competitiveness in swine gut or efficient immunomodulation validated by *in vivo* studies and maintaining relevant cell counts in the intestine by continuous feeding [138,139].

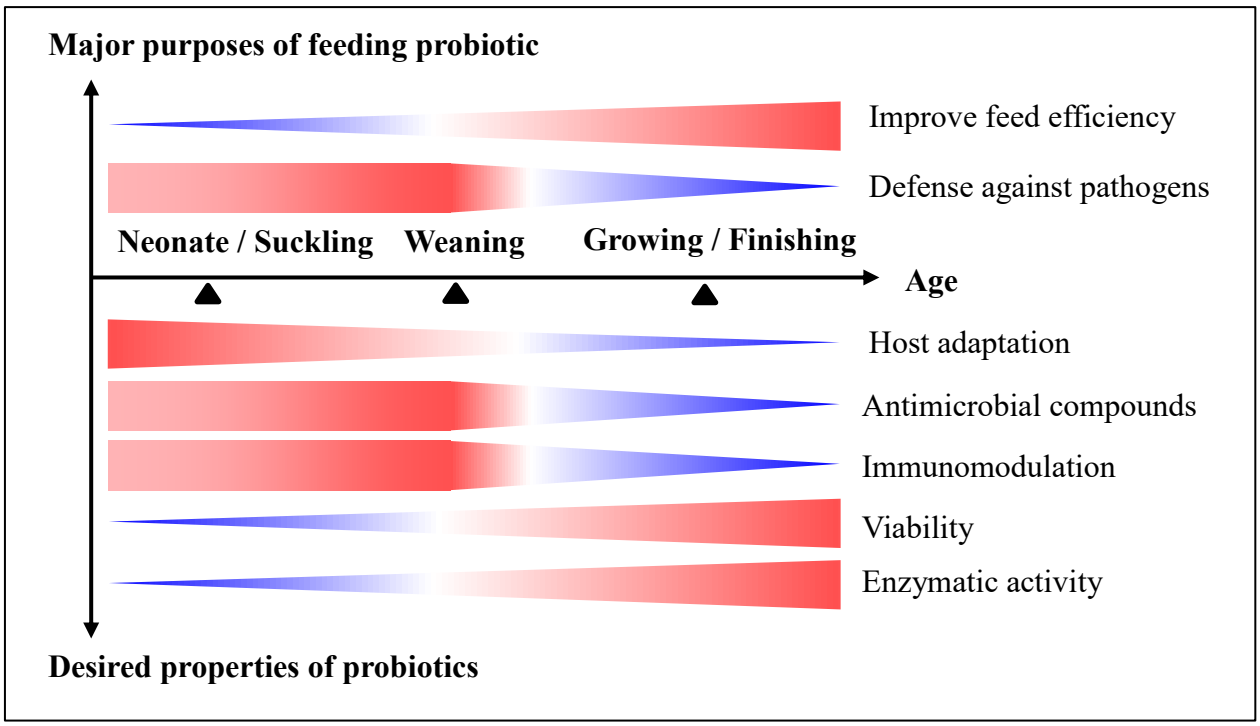
Grower / finisher pigs receive more protections from a mature immune system, which shifts the aim of applying probiotics toward improving growth performance [8,119,121]. Grower/finisher pigs also require higher doses of probiotic strains, thus, cost associated with preparation and stability of probiotic become more important. Preserving probiotics in a dry form is required for quick mixing with feed and maintaining a longer shelf life [151]. Spore forming probiotics can be added prior to pelleting of feed, which occurs at temperatures that are lethal to vegetative bacterial cells. Potent enzymatic activity is also a favorable trait of probiotics to aid feed digestion and enhancing growth performance of grower pigs [12]. Spore-forming *Bacillus* spp. have been referred to as outstanding commercial probiotics for growing pigs due to the excellent viability during drying processing, extended shelf life and potent phytase and cellulolytic activity [12,159]. Feed fermentation with lactic acid bacteria [160,161] is an alternative strategy to include probiotics while avoiding concerns related to drying and dry survival. Host-adapted lactobacilli are highly competitive in cereal fermentations; existing technology thus allows feed fermentations at a large scale [146,162,163]. Feed fermentation with probiotic lactobacilli improves the growth performance of pigs by combining the probiotic effects of lactic acid bacteria with the nutritional benefits of feed fermentation Note also that a longer period of probiotics supplying for grower / finisher pigs were suggested to achieve more improvemets in growth peformance [8,119,121,147].

2.6. Conclusion

In conclusion, effective probiotics are a promising solution to address the increasing concern of antibiotic resistance as a result of using antimicrobial growth promotors. The results of probiotic intervention trials in swine, however, are often inconsistent, which clearly demonstrates that “one

size does not fit all" and specific probiotic strains should be selected for specific applications. The critical review of selection criteria and particularly the consideration of the ecological origin of probiotics may guide future applications to use probiotics as an effective tool in swine production.

Figure 2.2. Major purpose of feeding probiotics at various growth stage and corresponding desired properties. The width (wide > narrow) and fill color (deep red > red > blue) of shapes represent degrees of emphasis for the purposes of feeding probiotic or desired properties of probiotics in the corresponding period.



Chapter 3. Identification and quantification of virulence factors of enterotoxigenic *Escherichia coli* by high-resolution melting curve quantitative PCR

3.1. Introduction

Post weaning diarrhea (PWD), especially enterotoxigenic *E. coli* (ETEC) related diarrhea, causes severe mortality and economical loss in the swine industry. Weaning imposes stress through the sudden change of diet and environment, and the interruption of immune protection from the sow's milk. Taken together, these stressors increase the susceptibility of piglets to diarrhea[50]. A high proportion of poorly digestible dietary protein following weaning also favours colonization of the intestine with pathogens. Antibiotics, including prophylactic antibiotics[1,164] and growth promoting antibiotics [50,165] are used to maintain gut health. The increasing concerns of antibiotic resistance development resulted in a ban of antibiotics as growth promoters in several jurisdictions [166,167], which makes control of post weaning diarrhea more difficult.

ETEC colonize the intestine by host-specific fimbriae that mediate adherence to receptors on the surface of the intestinal epithelium. After fimbriae-mediated colonization, ETEC strains produce toxins that disturb fluid homeostasis, thus causing severe diarrhea [50,60,78,168,169]. CFA/I, CFA/II and E8775 fimbriae mediate the attachment of ETEC to the human intestinal epithelium [170,171] whereas ETEC expressing K99 (F5), F41, F18, F6 (987P), and K88 (F4) fimbriae infect swine [78]. CFA/I fimbriae also act as a protective antigen which accelerate the immune response that protects the host from ETEC challenge [172]. Oral immunization with K88 fimbriae elicited a similar immune response as K88 fimbriae carrying ETEC infection in piglets; in both cases the immune response related to promotion of the gene expression of T cells producing IL-17 [173]. ETEC fimbriae thus play key roles in modulation of immune response which could supply more

strategies for ETEC prevention by vaccines or receptor analogues [152,172–174]. Both heat-labile (LT) and heat-stable enterotoxins (STa and STb) are detected in the ETEC related diarrheal samples [175–177]; few strains additionally carry Stx2e [178]. The profile of virulence genes in swine isolates of ETEC varies with the age of the animals and the geographical location; ETEC carrying K88 fimbriae are more frequent in neonate animals while ETEC carrying F18 fimbriae are more frequent in weanling pigs [60,78,169,177,179]. Moreover, vaccination of piglets with a recombinant K88/LT vaccine provided superior protection against ETEC K88 challenge when compared to vaccine with K88 or LT antigens alone [180]. The diversity of virulence factors of ETEC and role of fimbriae as targets for therapeutic intervention highlight the need of effective methods that differentiate fimbriae of ETEC [176,181–183].

PCR-based assays have become routine methods for rapid identification of bacterial pathogens [181,184–187]. High resolution melting (HRM) analysis is increasingly used to discriminate multiple targets in the same reaction [182,188,189]. HRM analysis distinguishes single base changes in target sequences [190]. HRM-PCR assays were established as simple, fast, and accurate methods for rapid identification of lactobacilli [191]. Multiplex PCR-HRM also simultaneously distinguished diverse virulence factors of Shiga-toxin producing *E. coli* (STEC) [188]. In addition to the sensitivity, HRM analysis was suggested as a cost efficient approach for differentiation of microorganism [189].

Quantitative HRM-PCR assays were first established to detect food adulteration, such as distinguishing admixtures to preparations of *Helleborus niger* for medical use [187] and detecting the presence of adulterations in basmati rice [192]. Relative quantification of template DNA with HMR-qPCR assays was initially based on the relationship between the level of normalised fluorescence at certain temperature and the proportion of the adulterant in the food or

pharmaceutical preparation [185,192–195]. A HRM-qPCR assay to simultaneously determine the relative proportions of four species of *Lactobacillus* in sourdough fermentation process achieved quantification by integration of the area of the melting peaks that are obtained by plotting the first derivative of the melting curve [196]. The peak area correlated linearly to the relative proportion of target sequences in a mixture of template DNA [196]. However, HRM-qPCR assays have not been developed for quantification of pathogens. This study therefore aimed to develop quantitative HRM methods to simultaneously identify and quantify five fimbriae types related to ETEC in swine, and to verify the reliability of the method in fecal samples obtained from weaned piglets.

3.2. Materials and methods

3.2.1. Microorganisms and growth conditions

Two K88 antigen positive ETEC strains, *E. coli* strains ECL13795 (O149; virotype STb: LT: EAST1: F4) and ECL13998 (O149; virotype STa: STb: LT: EAST1: F4: Paa) were supplied by *Escherichia coli* Laboratory (University of Montréal, QC, Canada) and incubated at 37°C overnight on Minca agar. Strains served as positive controls for the detection of K88 fimbriae, heat-labile toxin (LT), and heat-stable toxin (STa and STb) genes in qPCR and HRM-qPCR assays. Synthesized sequences and primers for qPCR and multiplex HRM-qPCR analysis.

Partial sequence of genes encoding the K99, F41, F18 and F6 fimbriae biosynthesis or subunit (Table 3.1) were synthesized by gBlocks® Gene fragment (Integrated DNA Technologies, San Diego, California, USA) for use as positive controls in HRM-qPCR assay. The sequences were obtained from GeneBank database (http://www.ncbi.nlm.nih.gov) (Table 3.2). The specificity of primers was confirmed by PCR reaction with positive controls and DNA isolated from feces samples. For use as standards in HRM-PCR analysis, positive controls were amplified by PCR with specific primers (Table 3.2) and purified by agarose gel electrophoresis. The concentration

of the amplicons was determined by nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 260 nm and amplicons were diluted to 10^{10} gene copies / μL.

Table 3.1. Synthetic DNA probes as positive controls for K99, F41, F18, and F6 fimbriae gene sequences for qPCR and multiplex HRM-qPCR analysis

Target (gene bank accession)	Target gene	Sequence	Location	Size (bp)
K99 fimbriae (X05797.1)	*E. coli* genes fanA and fanB involved in biogenesis of K99 fimbriae	GCATAAAACTCTGGTTCTTCTTGGCTGTTTATTTTTTTTTT CTATATGTTCAGTGTGTTATTTATACTCTTCCCTTTATTTT TGTTTTTTTTATGCCATATAATTCAATCAGCAGAGATGAT TGGGATCATAAAAATGTCACTTGAGGGTATATGCGATCTT TTAATAAAGATGAATACTTGTTCAGGGAGAAACTTGGTT ATCTTGTGAAAGGAATGGTTAAAGCAAGGTGCTTCCAAT TATTAGTGGAGTTATCAAGTATACGTAGTTCTAGGG	314-589	276
F41 fimbriae (X14354.1)	*E. coli* fimbriae F41a gene for F41 fimbriae subunit	ACAATTGGGATGACCTCAGTCACAGCAACTATACTTCTGC AAATAAGGCATCTTATCTCTCTTATGGATCTGGTGTTTCT GCAGGTAGTACTTTAGTTATGAATTTAAATAAGGATGTTG CGGGTCGACTTGAATGGGTGG	916-1056	141
F18 fimbriae (KM260195.1)	*E. coli* isolate HDG_U113 FedA precursor (fedA) gene	AACACAGGGGCAGGAGGTTAAGGCGTCGAATAGCACTGT AAGTTTCGATGCATCAAAAGCAACTACGGAAGGTTTCAA ATTTACTGCTCAACTGAAAGGTGGTCAAACCCCGGGTGA CTTCCAGGGGGCAGCGGCTTACGCGGTTACTTACAAG	357-510	154
F6 (987P) fimbriae (M35257.1)	*E. coli* fimbriae 987P subunit gene	ACTAAATATTTAGTTCCAGCCTCCAATGATACTAGTGCAT CAGGAGTTGGCGTATACATTCAGGACAACAACGCCCAGG CTGTGGAAATTGGTACTGAAAAAACTGTACCTGTGGTATC AAATGGCGGATTAGCTCTTTCAGACCAAAGTATTCCACTG CAAGCATACATCGGAACCACCACAGGGAATCCTGA	574-767	194

Table 3.2. Primers used for qPCR and multiplex HRM-qPCR analysis

Target gene	Sequence (5'--3')(name)	Size (bp)	T_A (°C)[A]	Reference
K99 fimbriae (*fan A*)	CACTTGAGGGTATATGCGATCTT (K99 F) GACCTCAGTCACAGCAACTATAC (K99 R)	92	62	This study
F41 fimbriae Sub-unit A	GACCTCAGTCACAGCAACTATAC (F41 F) CGACCCGCAACATCCTTATT (F41 R)	110	62	This study
F18 fimbriae (*Fed A*)	GGAGGTTAAGGCGTCGAATAG (F18 F) CCACCTTTCAGTTGAGCAGTA (F18 R)	90	62	This study
F6 fimbriae (*Fas A*)	GTTCCAGCCTCCAATGATACT (F6 F) GAAAGAGCTAATCCGCCATTTG (F6 R)	128	62	This study
K88 fimbriae (*fae G*)	GCACATGCCTGGATGACTGGTG (K88 F) CGTCCGCAGAAGTAACCCCACCT (K88 R)	439	63	[197,198]
E. coli (Universal stress protein A)	CCGATACGCTGCCAATCAGT (UspA F) ACGCAGACCGTAGGCCAGAT (UspA R)	884	66	[184,197]
Heat-labile toxin	CCGTGCTGACTCTAGACCCCCA (LT F) CCTGCTAATCTGTAACCATCCTCTGC (LT R)	480	68	[197,199]
Heat-stable toxins a	ATGAAAAAGCTAATGTTGGC (STa F) TACAACAAAGTTCACAGCAG (STa R)	193	65	[186,197]
Heat-stable toxins b	TGCCTATGCATCTACACAAT (STb F) CTCCAGCAGTACCATCTCTA (STb R)	113	60	[197,200]

[A] T_A, primer annealing temperature.

3.2.2. Animals and growth environment

To validate the HRM-qPCR assay with animal samples, samples were collected at the Swine Research and Technology Centre (Edmonton, AB, Canada); animals were housed following the guidelines of the Canadian Council on Animal Care and trials were approved by the University of Alberta Animal Care and Use Committee. Samples from healthy animals were collected from crossbred weaned pigs (Duroc × Large White, aged 6-7 weeks) that were housed under standard sanitary conditions. Fecal samples were collected from 16 pigs housed in a clean room; 8 samples were collected from pigs with a fecal score of 5-6 at least once (diarrhea episode), and 8 samples were collected from pigs with feces score of less than 5 (healthy control). Samples from animals with diarrhea were collected from crossbred weaned pigs (Duroc × Large White, aged 6-7 weeks)

that were housed under poor sanitary conditions to induce diarrhea. Briefly, the housing was not cleaned before the piglets were moved to the facility and pooled feces from the sow herd were spread on the flooring twice: the day before new pigs were introduced and 1 week later. Feces was scored visually for consistency from 1 (solid feces) to 8 (watery diarrhea). Fecal samples were obtained from 14 pigs with a score 7 or greater for more than 3 three days.

3.2.3. Sample collection and DNA extraction

Bacterial DNA was extracted from bacterial cultures with the DNeasy Blood & Tissue Kit (QIAGEN, Waltham, Massachusetts, USA). Fresh fecal samples were collected directly from rectum after stimulating animals to defecate. Fecal samples were placed in sterile plastic bags and stored at -80°C. Frozen samples (0.2g) were homogenized with ASL buffer and heated at 95 °C for 15 min to lyse cells and the supernatant was isolated by centrifuging at 18,800× g for 1 min. DNA extraction from feces samples followed the manufacturer instruction of QIAamp DNA stool minikit (QIAGEN). Template DNA was diluted to a concentration of 50 mg / L.

3.2.4. Identification and quantification of ETEC fimbriae genes by individual / multiplex HRM-qPCR

All HRM-qPCR reactions were performed using a Rotor-Gene Q (QIAGEN) HRM-thermo cycler and Type-it HRM Kit (QIAGEN) Primers targeting five different porcine ETEC fimbriae genes (K99, F41, F18, F6 and K88) were designed with nearly identical annealing temperature (62 °C to 63 °C) (Table 3.2) to allow amplification in multiplex PCR reaction. HRM-qPCR reactions contained 12.5 µL 2× HRM Master Mix, 2 µL template DNA for individual reaction or 3 µL for multiplex reaction, 700 nM primers for individual reaction and 200 nM per target for multiplex detection to a final volume of 25 µL. The optimized PCR conditions were 5 min initial denaturation at 95 °C, 45 cycles of denaturation at 95 °C for 10s, annealing at 62 °C for 30s and extension at

72 °C for 25s. During the HRM stage, temperature increased from 65 °C to 95 °C at the speed of 0.1 °C/ step and held for 2s at each step.

qPCR reactions with single amplicons were calibrated by using serial 10-fold dilutions of positive controls to obtain standards containing 10^2 to 10^8 gene copies / μL as template. Multiplex HRM-qPCR combined absolute quantification of all template genes with relative quantification of the proportion of individual genes. A standard curve for the gene copy number of all target genes in multiplex amplification was established from serial 10-fold dilutions of positive controls to obtain standards containing 2×10^2 to 2×10^8 gene copies / μL of each of the five targets. The relative quantification was conducted on the basis of the linear correlation between the relative areas of the respective melting peaks to the relative proportion of specific target sequences in the mix of template DNA. The raw melting curve was deconvoluted by PeakFit software (Systat software Inc., San Jose, California, USA) using AutoFit Baseline and AutoFit Peaks I Residuals methods. To establish the calibration curves, five to seven different ratios of target were mixed with known concentration mixture DNA template and the ration of target to total DNA was plotted against the corresponding proportion of melting peak area to the total peak area. The calibration equations of the five fimbriae was verified by varying the percentage of the target fimbriae gene sequence in a template mixture containing the gene fragments of the four other fimbriae with identical gene copy number.

3.2.5. Quantification of *uspA* and toxin genes by SYBR Green based qPCR analysis

Primers used for *uspA*, STa, STb and LT toxins quantification are listed in Table 3.2. qPCR was performed on a 7500 Fast real-time PCR system (Thermo Fisher Scientific) using MicroAmp Fast Optical 96-well reaction plate (Thermo Fisher Scientific). qPCR reactions contained 10 μL QuantiFast SYBR green master mix (Thermo Fisher Scientific), 2 μL (10 μM) primers, 1 μL

template DNA, and water to a final volume of 20 µL. PCR conditions were as follows: initial denaturation 5 min at 95°C, 40 cycles of denaturation at 95°C for 30s, annealing at corresponding temperature (Table 3.2) for 30s and followed by 30 extension at 72°C. At the melting stage, temperature increased with a speed of 0.5 °C/10s from 55 to 95°C. To calibrate qPCR assays, target genes were amplified from chromosomal DNA of *E. coli* ECL13998, purified by agarose electrophoresis, and the concentration was determined by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The calibration of the qPCR assays was performed on the same instrument platform, with the same reagents and on the same day as the respective qPCR or HRM-qPCR assays.

3.2.6. Statistical analysis

Experiments were conducted at least in triplicates and results were reported as means ± SEM. Data analysis was performed with Linear Regression model (PASW Statistics 18.0, Quarry Bay, HK, China) and $p \leq 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Identification of genes encoding ETEC fimbriae by multiplex HRM-qPCR

A multiplex HRM-qPCR assay was developed for the simultaneous identification and quantification of five genes of fimbriae that mediate adhesion of ETEC to swine intestinal cells. HRM-qPCR separated the melting peaks of K99, F41, F18, F6 and K88 fimbriae gene amplicons with 1 to 2°C difference (Figure 3.1, Table 3.3). The melting curve results also indicated that melting temperatures of amplicons shift by up to 1°C in multiplex PCR compared to the individual reactions. All melting temperatures obtained in multiplex assays were higher than that of individual amplicons; this difference was more pronounced for the genes encoding F6 and K88 fimbriae (Figure 3.1). These results conform to the stabilizing effect of Evagreen, the fluorescent

dye used in this HRM assay, on double stranded DNA. Tm values of dsDNA increased with the increasing dye concentration and decreasing amplicon concentration in most DNA binding dyes [201,202]. The concentration of free Evagreen increases during melt curve analysis as the dye is released from double stranded DNA. During the melting curve stage of multiplex HRM-qPCR, dye release from the lower melting K99, F41 and F18 amplicons increased the dye concentrations and hence may have increased the binding strength and Tm shift for the higher melting F6 and K88 amplicons. With equal starting concentration of five targets sequences, amplified in multiplex conditions, the area of the five melting peaks differed substantially; the peak height of K99 was the lowest while K88 was the highest. As Evagreen showed equal preference for GC- or AT- rich amplicons, this results may indicate the lower affinity of Evagreen towards shorter double-strand DNA [203,204]. The size of the amplicons ranged from 90 to 439 bp (Table 3.2). Due to the preferential binding to the dye, the peak area and height increased with increasing amplicons length in multiplex reaction. (Figure 3.1). The efficiencies of PCR with the 5 primer pairs did not differ substantially (Table 3.3), minimizing any additional effect of PCR efficiency on the area of the melting peaks.

Table 3.3. Melting temperature and calibration parameters for multiplex HRM-qPCR detection of K99, F41, F18, F6 and K88 fimbriae genes

	K99	F41	F18	F6		K88
Tm (°C)	74.7±0.06	76.5±0.12	77.6±0.35	79.5±0.78		80.5±0.15
E[A]	1.87	1.74	1.7	1.85		1.84
r_1^{2}[B]	0.99	0.99	0.99	0.99		0.99
DNA range	1-20%	0.2%-16.7%	1%-100%	0.3%-4.8%	4.8%-60%	4.8%-20%
Slope[C]	2.96	2.74	2.39	0.48	1.44	1.49
r_2^{2}[D]	0.97	0.97	0.98	0.97	0.99	0.97

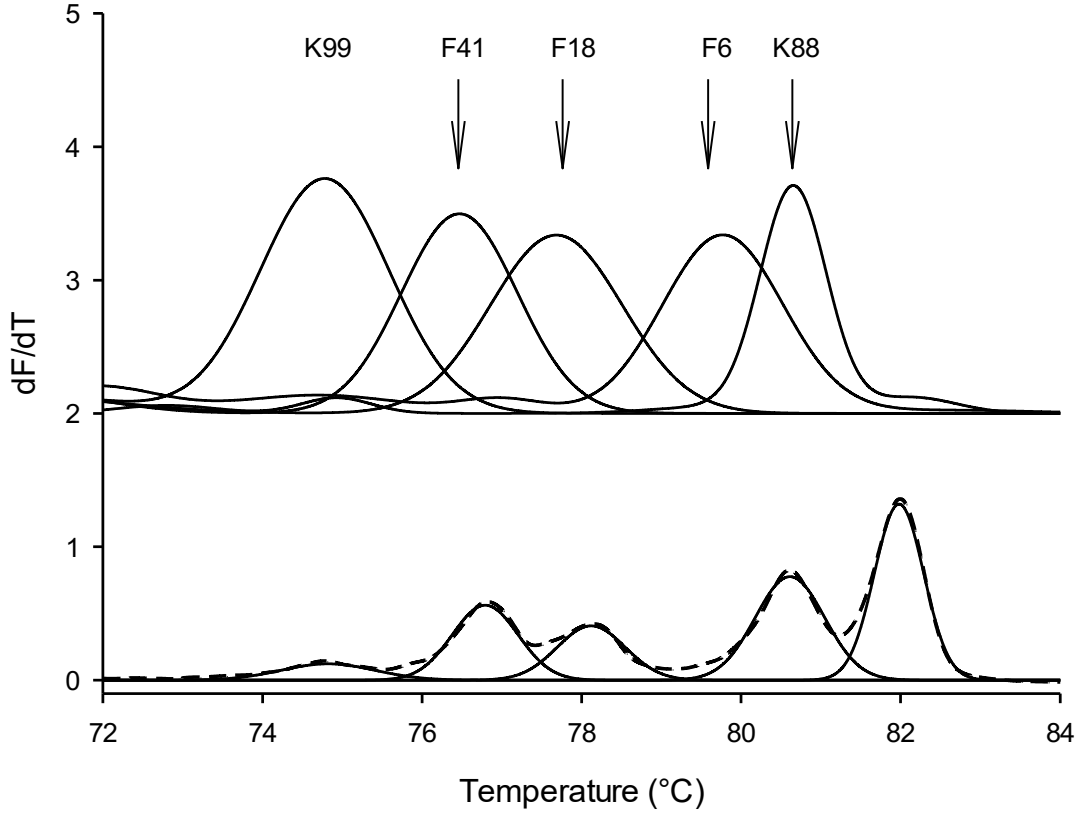
[A], primer efficiency determined by individual PCR.

[B], r_1^2, correlation coefficient for standard curve determined by individual qPCR.

[C], Slope for calibration curve correlating the area of the melting peaks area to the proportion of the template DNA

[D], r_2^2, correlation coefficient for standard curve correlating the area of the melting peaks to the proportion of the template DNA

Figure 3.1. Melting curve of target sequence from positive controls by individual HRM-qPCR (top), melting curve of PCR products amplified from a mixed positive control including five different fimbriae gene sequence by multiplex HRM-qPCR (bottom, dotted) and the corresponding reprocessed melting curve by PeakFit software (bottom, solid).

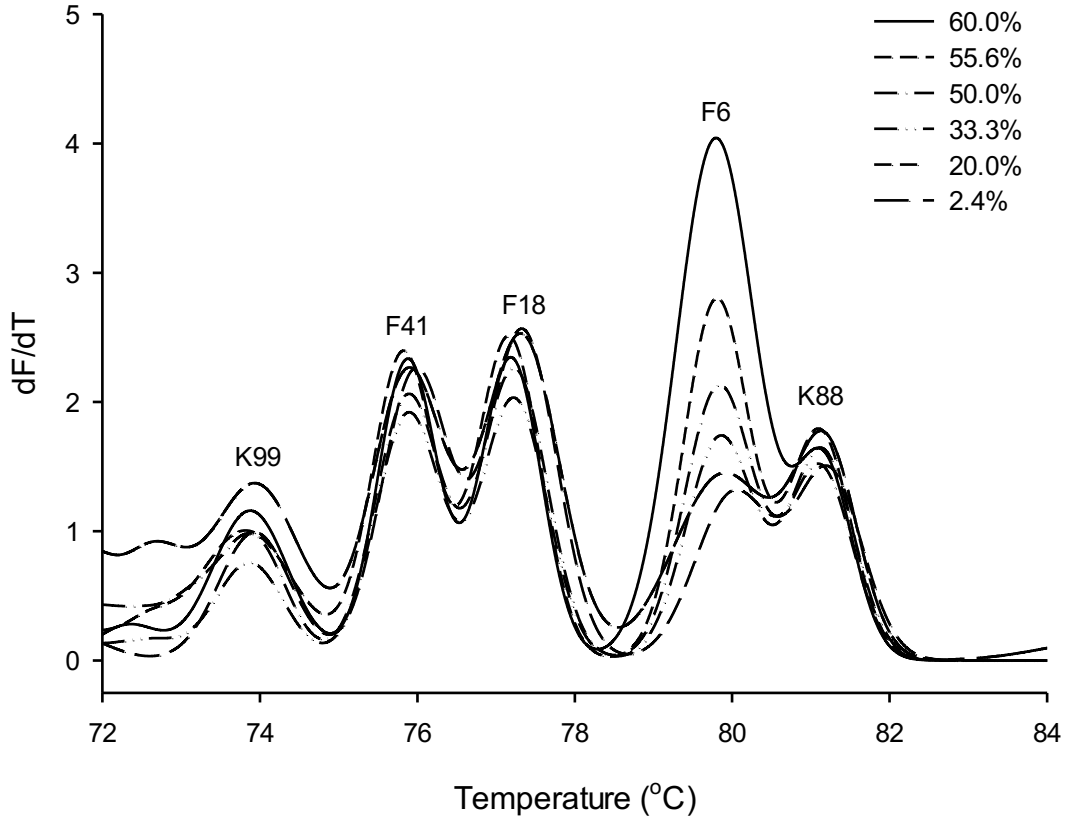


3.3.2. Quantification of genes encoding ETEC fimbriae by multiplex HRM-qPCR

To establish calibration curves for individual target genes in a mixture of all five genes, all five genes were mixed in equal molar concentrations and the proportion of one of the five was successively reduced. Template DNA containing fixed amounts of four target genes and a variable amount of one target gene were analyzed by HRM-qPCR (Figure 3.2). Calibration curves were established by correlating of the relative area of the melting peaks to the proportion of DNA in the

mix of template DNA (Table 3.3). The calibration range was chosen to cover the content of target DNA in fecal samples (see below). The lowest detection limit was achieved for genes encoding F41 fimbriae, which were detected when their relative proportion exceeded 0.2% of total gene copy numbers. Two calibration curves were established for the gene encoding F6 fimbriae; one calibration curve covered the relative DNA content of 0.3 to 4.8%, a second calibration curve covered the relative DNA content of 4.8 to 60% (Figure 3.2 and Table 3.3). The r^2 of all regression equations was greater than 97% (Table 3.3).

Figure 3.2. Calibration of F6 by changing the DNA range from 2.44% to 60% in the total gene copy number. Gene copy number of K99, F41, F18 and K88 was constant at 1×10^{10}. The percentages of F6 melting peak area and DNA range were plotted to establish the calibration equation parameters.



3.3.3. PCR quantification of genes encoding ETEC fimbriae in fecal samples

Fecal samples collected from 30 weaning pigs were analysed to assess the applicability of the multiplex HRM-qPCR methods. Samples were obtained from 14 weaning piglets where persistent diarrhea was induced by poor diet and poor sanitary conditions, and from 16 weaning pig that were kept in normal conditions and remained healthy (n=8), or experienced diarrheal episodes (n=8). ETEC fimbriae genes and toxins genes were quantified to determine if ETEC infection contributed to the persistent diarrhea or diarrheal episodes. HRM-qPCR distinguished amplicons of genes encoding ETEC fimbriae in fecal samples (Figure 3.3). Multiplex HRM-qPCR analysis detected all five ETEC fimbriae in all fecal samples collected from animals with diarrhea (Table 3.3); moreover, the gene copy number of ETEC fimbriae types exceeded the detection limit in the most of fecal samples collected from healthy animals or animals with diarrhea episodes (Figure 3.3, Table 3.4). The gene copy numbers of K99, F41 and K88 fimbriae were below the detection limit of the multiplex HRM-qPCR assay in several samples (Table 3.4). The area of melting peaks demonstrated that F18 was the predominant fimbriae type in animals with persistent diarrhea (Figure 3.3, Table 3.4) but not in healthy animals. In animals with persistent diarrhea, high copy numbers of genes encoding fimbriae of ETEC were detected. Moreover, gene copy numbers of ETEC fimbriae did not differ from the gene copy number of *uspA*, which is present in all strains of *E. coli*, or the copy number of the gene encoding the STb toxin (Table 3.4). These results indicate that a majority of *E. coli* in the fecal samples were ETEC. However, in pigs with diarrheal episodes, or in healthy pigs, numbers of *E. coli* exceeded the numbers of ETEC more than 10,000-fold (Table 3.4).

Figure 3.3. Melting curves of mixed positive control (top, dotted) and the same melting curve reprocessed by PeakFit software (top, solid), melting curve of a swine diarrhea feces sample (bottom, dotted) and the corresponding reprocessed melting curve. The two curves are offset by 2 dF/dT.

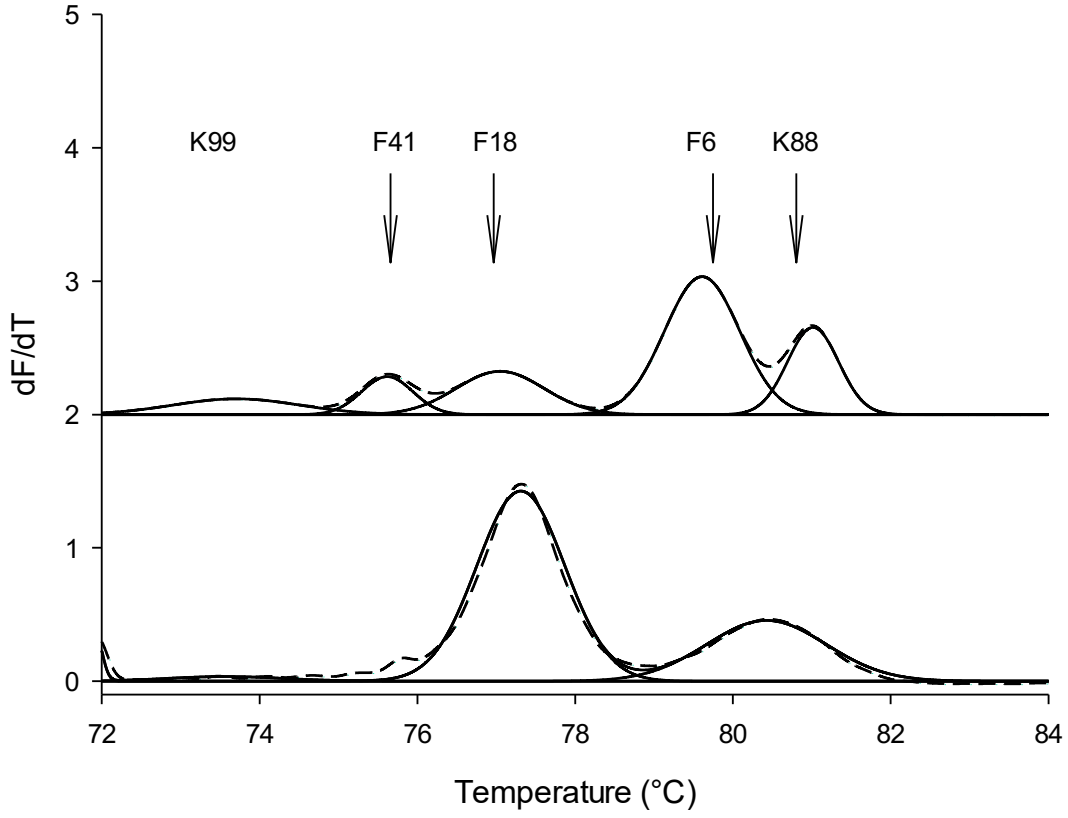


Table 3.4. Gene copy number of ETEC fimbriae genes, total ETEC fimbriae, *UspA* and toxins genes of diarrhea observed, and health piglets detected by qPCR and HRM-qPCR methods. Data are presented as means of the $\log_{10}$ number of gene copies/g wet feces ± SEM in positive samples[A]

Target genes	Diarrhea [B] Observations (n = 14)		Potential Diarrhea [B] Observations (n = 8)		Healthy animals (n = 8) [B]	
	Individual qPCR	Multiplex HRM-qPCR	Individual qPCR	Multiplex HRM-qPCR	Individual qPCR	Multiplex HRM-qPCR
K99 fimbriae (*fan A*)*	5.85±0.14 (14/14)[D]	7.52±0.13 (14/14)	6.35±0.04 (8/8)	3.99±0.28 (6/8)	6.37±0.05 (8/8)	3.97±0.10 (5/8)
F41 fimbriae Subunit A*	6.67±0.07 (14/14)	7.87±0.13 (14/14)	4.46+0.07 (8/8)	3.62±0.16 (6/8)	4.67±0.14 (8/8)	4.47±0.43 (7/8)
F18 fimbriae (*Fed* A)	8.51±0.17 (14/14)	8.39±0.22 (14/14)	5.03±0.09 (8/8)	5.07±0.08 (8/8)	4.89±0.09 (8/8)	5.04±0.14 (8/8)
F6 fimbriae (*F*as A)*	6.60±0.06 (14/14)	7.81±0.12 (14/14)	4.43±0.10 (8/8)	3.90±0.09 (8/8)	4.28±0.10 (5/8)	4.25±0.12 (7/8)
K88 fimbriae (*fae G*)	7.15±0.07 (14/14)	7.51±0.14 (14/14)	5.74+0.07 (8/8)	4.10±0.23 (8/8)	5.52±0.05 (8/8)	3.60±0.18 (6/8)
ETEC [C]	N/A	7.99 ± 0.17^{a} (14/14)	N/A	4.63 ± 0.06^{b} (8/8)	N/A	4.68 ± 0.13^{b} (8/8)
Universal stress protein A	9.21±0.17 (14/14)	N/A	8.92±0.20 (8/8)	N/A	8.80±0.19 (8/8)	N/A
Heat-labile toxin	4.24 ± 0.23^{a} (9/14)	N/A	$< 3^{b}$	N/A	$< 3^{b}$	N/A
Heat-stable toxins a	6.22 ± 0.21^{a} (14/14)	N/A	5.11 ± 0.71^{a} (8/8)	N/A	3.02 ± 0.01^{b} (8/8)	N/A
Heat-stable toxins b	7.80 ± 0.18^{a} (14/14)	N/A	5.03 ± 0.09^{b} (8/8)	N/A	4.89 ± 0.09^{b} (8/8)	N/A

[A] the detection limit of individual qPCR was 10^4 copies / g wet feces; the detection limit of multiplex HRM-qPCR was 10^5 copies / g wet feces.

[B] Diarrhea observation corresponds to fecal scores of 7 or higher for more than 3 days; potential diarrhea observation corresponds to fecal scores ranging from 5 to 6 at least once; healthy animals corresponds to fecal scores of less than 5;

[C] ETEC was calculated by the CT values and standard curve of HRM-qPCR detection for the feces samples, including the amplification of K99, F41, F18, F6, and K88. ETEC was calculated as the sum the gene copy numbers of all five fimbriae genes as obtained from the CT value of fecal samples by multiplex HRM-qPCR;

[D] number of positive samples / numbers of total samples.

N/A, not analyzed.

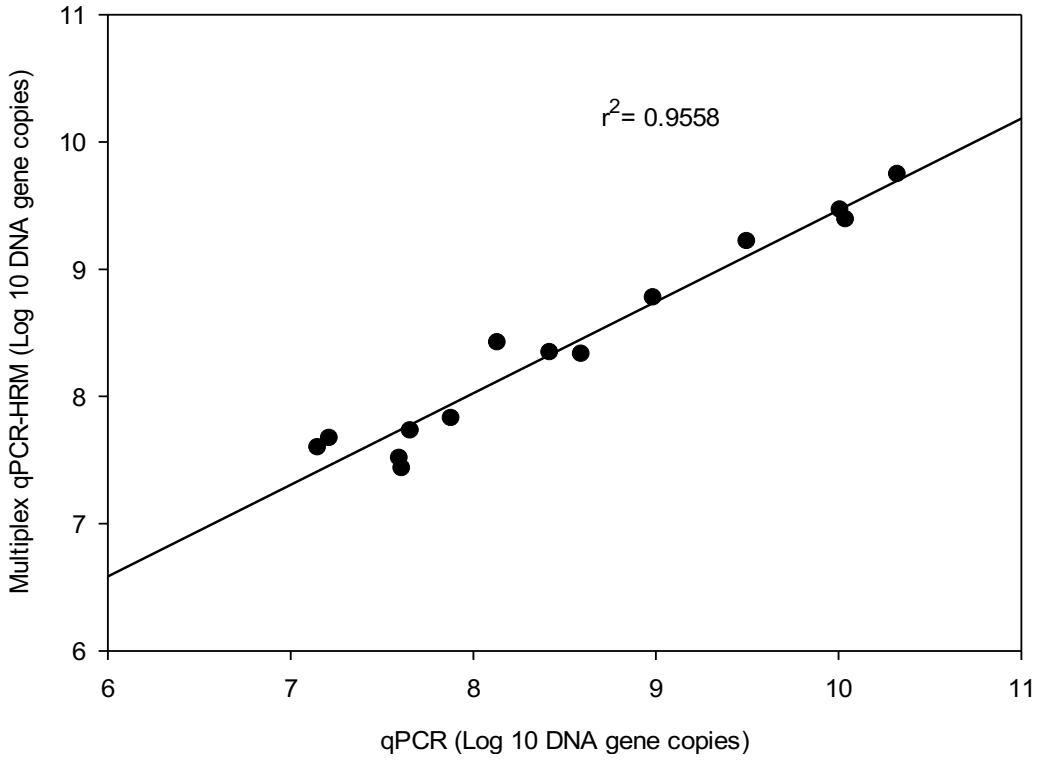
* means results detected by qPCR and HRM-qPCR are significantly different ($P < 0.05$).

Data in the same row (ETEC, Universal stress protein A, Heat-labile toxin, Heat-stable toxin a and Heat-stable toxin b) that do not share a common superscript are significantly different ($P < 0.05$)

3.3.4. Comparison of multiplex HRM-qPCR to quantification with individual qPCR reactions

To verify the reliability of multiplex HRM-qPCR, results of HRM-qPCR analysis were compared to specific qPCR assays as reference method for quantification of DNA. Multiplex HRM-qPCR identified fimbriae types without false positive results (Table 3.4). Multiplex HRM-qPCR detected the target DNA in fecal samples with a detection limit 10^5 copies / g wet feces while the detection limit of qPCR assays ranged from $10^3 – 10^4$ gene copies / g wet feces. The consistency of qPCR and multiplex HRM-qPCR quantification was analysed by regression analysis (Figure 3.4). Correlation of data obtained for quantification of the most abundant F18 fimbriae revealed a r^2 of 0.9558 ($P < 0.001$), demonstrating a high consistency between the two methods. However, the gene copy number obtained by multiplex HRM-qPCR analysis was inconsistent with the gene copy number obtained by qPCR for K99, F41 and F6 ($P < 0.05$). An r^2 of 0.3572 was obtained when data for all five fimbriae were used for the correlation analysis. The difference between qPCR and multiplex HRM-qPCR analysis likely reflects overestimation of low abundance target genes by HRM-qPCR.

Figure 3.4. Scatter plot and regression analysis between the Log_{10} gene copy number of F18 fimbriae gene measured by qPCR and multiplex HRM-qPCR methods (n=14)



3.4. Discussion

The diversity of virulence factors of swine ETEC and the occurrence of hybrid virotypes necessitates identification of pathogenicity for control and treatment of post weaning diarrhea in swine production [50]. F18 and K88 positive ETEC strains are the most widespread cause for the *E.* coli related post weaning diarrhea and edema disease in piglets [168,205]. *E. coli* with F18 fimbriae cause diarrhea in weaned piglets. Neonatal pigs lack receptors for F18 fimbriae and ETEC with K88 fimbriae typically cause diarrhea in nursing piglets [50,205]. More than 70% of F18 fimbriae positive ETEC strains isolated from diarrheal pigs in the US produce STa and STb. In contrast, strains expressing K88 fimbriae usually produce LT and STb while STa production is infrequent [82,169,206]. Active immunization or passive immunization are used to protect

neonatal and weaning pig since they are highly susceptible to ETEC infection [50]. Successful immunisation strategies target the different fimbriae addition to the toxins, and hence require knowledge on the fimbriae that are related to disease development [207]. Oral immunization with recombinant vaccines such as recombinant K88/LT vaccine [180] or recombinant K88/K99/F6/F41/F18 fimbriae proteins [174] was more advantageous in stimulation of systematic and mucosal immunity [180]. Strategies that employ receptor decoys to prevent binding of specific fimbriae types to the glycan receptor on the surface of the intestinal mucosa provide an alternative therapeutic option to prevent ETEC-induced diarrhea in young pigs. However, different fimbriae bind to different glycan receptors and hence require the use of different therapeutic oligosaccharides [208]. For example, EPS from L. reuteri was shown to prevent adhesion of K88 ETEC but its effect on ETEC expressing other fimbriae remains to be demonstrated [174]. In brief, several therapeutic options for diarrheal disease in piglets including specific recombinant vaccines target bacterial fimbriae and hence depend on diagnostic tools that identify the fimbriae type associated with the diarrheal pathogen. Because several ETEC strains expressing different fimbriae may be present simultaneously, diagnostic tools should be able to identify fimbriae types that are most abundant and hence most relevant for disease development.

HRM analysis discriminates sequence variations between amplicons by determination of high resolution melting curves with a precision of 0.1°C [185]. Compared to the individual qPCR analyses, multiplex HMR-qPCR assays are a suitable, cost-effective and high-throughput strategy for qualitative or quantitative analysis of pathogenic *E. coli* [185]. Previous studies employed HRM-qPCR to confirm the presence of *E. coli* in an ETEC-challenged small intestinal segment perfusion model [152]. Differentiation between *E. coli* and other bacterial taxa was achieved by HRM analysis of amplicons of 16S rRNA genes; confirmation of strain identity was provided by

qPCR analysis of strain-specific virulence factors [152]. Additionally, a multiplex HRM-PCR platform was established to discriminate among virotypes of *E. coli* on the basis of the presence or absence of 7 genes encoding virulence factors [209]. However, the choice of virulence factors that were included in the assay did not encompass those genes that are required to differentiate between different ETEC in swine. This study employed HRM-qPCR to differentiate between swine-associated ETEC strains expressing 5 different types of fimbriae. PCR primers were selected to obtain amplicons separated by 1 – 2°C, which was sufficient to differentiate the genes in samples containing all five genes.

The multiplex HRM-qPCR assay established in this study not only identified genes encoding virulence factors of swine-associated ETEC, it also quantified their relative abundance. Most other quantitative multiplex HRM-qPCR methods achieve quantification of two or more amplicons based on the normalised fluorescence level [187,193]. Quantification methods developed for basmati rice adulteration, however, allowed confident detection and quantification only when the percentage of adulteration was more than 15% [192–195]. Quantification of multiple amplicons based on the area under the melting peak was first developed to quantify *Lactobacillus* spp. in sourdough [196]. Robust identification and integration of melting peaks that are obtained by the df/dT derivative of the melting curves is achieved with standard chromatography software [196]. Reprocessing the melting curves with chromatography software also increased the accuracy of quantification by reducing the signal-to-noise ratio. The quantification method allowed accurate detection ($R^2 > 0.98$) of multiple *Lactobacillus* species when the corresponding DNA content was more 0.2% of the total DNA [196]. This previous method used a single primer pair to obtain amplicons of 16S rRNA genes that differ in their melting temperature, therefore, concerns related

to primer annealing and PCR efficiency of multiple primers for amplification of multiple genes were not addressed [196] .

The multiplex HRM-qPCR developed in this study adopted the relative quantification method based on melt peak area [196] but applied five specific primer pairs rather than a universal primer pair. Primers were selected to obtain identical primer annealing temperatures but amplicons that differ in the melting temperature. Moreover, PCR conditions of multiplex amplification were optimised to achieve similar amplification efficiency. Because primer design for HRM-qPCR is constrained by the necessity of obtaining 5 primer pairs with the same annealing temperature but amplicons having different melting temperatures, the PCR efficiency was not further optimized. The method developed in this study detected genes encoding target fimbriae if their proportion of the total target DNA exceeded 0.2%. The relative quantification results of multiplex HRM-qPCR were comparable to individual qPCR for the predominant fimbriae type but HRM-qPCR provided a higher relative proportion for low abundance targets when compared to conventional qPCR. Although qPCR is considered the "gold standard" for sequence-specific quantification of DNA, discrepancy with other quantitative methods were also observed in comparison of qPCR to other methods for quantification of DNA, e.g. microarray analysis or high-throughput sequencing [210,211]. Moreover, culture-based methods may be superior to DNA-based methods for quantification of viable *E. coli* in food and intestinal samples [212].

3.5. Conclusion

As specific receptors on the host epithelia cells mediates the adhesion and colonization by ETEC, the susceptibility of swine to ETEC infection is determined by animal lineage and age [61]. Enterotoxins typically occur combined with specific serogroups and fimbriae [169]. The multiplex HRM-qPCR assay developed in this study distinguished five different fimbriae gene by optimizing

the combination of primer pairs and reaction conditions. Moreover, the relative quantification based on melt curve area confirmed the prevalence of F18 in weaned pigs and indicated that ETEC was associated with persistent diarrhea in weaning piglets. Accurate diagnosis of major fimbrial antigens and virulence determinants by multiplex HRM-qPCR may thus provide the basis for disease prevention [50], and to develop treatments targeting ETEC on the basis of their fimbriae type [197].

Chapter 4 Impact of probiotic *Lactobacillus* spp. on autochthonous lactobacilli in weaned piglets

4.1. Introduction

Weaning piglets undergo abrupt changes in diet, social and environmental conditions at weaning, and are therefore susceptible to enteric pathogens including *Escherichia coli* and *Clostridium perfringens* [176,213]. Feed antibiotics have been used to manage pig gut microbiota, however, these also contribute to development of antibiotic resistance [214]. Many jurisdictions restrict antibiotics to therapeutic use, therefore prohibiting the prophylactic use of antibiotics and antimicrobial growth promoters. Probiotic bacteria are an alternative to prophylactic antibiotics to prevent diarrheal disease in swine [215,216]. The efficacy of probiotic *Lactobacillus* spp. to animal health has been extensively documented [215].

Selection criteria for identification of probiotic lactobacilli are currently lacking. It remains unclear whether health-promoting activities are strain or species specific, or whether these characteristics are generally shared among *Lactobacillus* spp. Moreover, the genus *Lactobacillus* has an exceptional phylogenetic and physiological diversity. The genus encompasses 24 taxonomic groups, each of these represents a diversity that is typically observed in a bacterial genus [217]. Lifestyles of *Lactobacillus* spp. were distinguished as ‘free-living’, ‘nomadic’ or ‘host-adapted’ [131], based on the increasing availability of large-scale analysis of individual *Lactobacillis* species by large-scale comparative genomics in combination with ecological studies [44,131]. Host-adapted lactobacilli have a stable association with one or more species of vertebrate or insect hosts; free living lactobacilli are adapted to environmental or plant-associated habitats; nomadic lactobacilli combined a free living lifestyle with the ability to temporarily persist in diverse animal or insect hosts [131]. This concept provides a rationale for selection of probiotics and an ecological

perspective to interpret observations in human and animal trials. Autochthonous *Lactobacillus* spp. are present in high cell counts throughout the GI tract of pigs soon after birth and colonize the gastric epithelium [218,219]. Members of the *L. reuteri*-, *L. delbrueckii*- and *L. salivarius*-groups are dominant; the species *L. reuteri* and *L. amylovorus* are most frequently isolated [215,220,221]. *L. reuteri* is further differentiated in host-adapted lineages that colonize the intestine of swine, chicken, rodents, and humans [44,222,223].

This study aimed to determine whether host-adapted lactobacilli exhibit superior survival during gastrointestinal transit relative to nomadic and free-living organisms, and to characterize the impact of probiotic lactobacilli on autochthonous lactobacilli. The study employed *L. reuteri*, *L. casei*, a species with a nomadic lifestyle without niche specialization [131,224] and *L. fermentum*, a species with a free-living lifestyle associated with plant material or environmental habitats [131]. The comparison of a reutericyclin-producing strain of *L. reuteri* and its isogenic reutericyclin-negative derivative [225,226] was used to assess the impact of specific antimicrobial metabolites on autochthonous lactobacilli [23].

Vegetative cells of probiotic cultures are generally freeze-dried for use in food/feed applications (Ross et al., 2005) but can be alternatively applied in feed fermentations [216,227]. The study therefore delivered probiotic cultures as freeze-dried preparations or as fermentation organisms in fermented feed.

4.2. Materials and methods

4.2.1. Microorganisms and growth conditions

The reutericyclin producing *L. reuteri* TMW1.656 and the reutericyclin negative mutant *L. reuteri* TMW1.656Δ*rtcN* [225] and two commercial probiotics, *L. casei* K9-1 and *L. fermentum* K9-2 were routinely grown on MRS5 agar [228] at 37°C under anaerobic conditions. *L. reuteri*

TMW1.656 is a rodent-lineage representative of the vertebrate-host adapted species *L. reuteri* with documented probiotic activity in swine [216]; *L. casei* has been attributed a nomadic lifestyle and *L. fermentum* is associated with environmental habitats [131]. Food grade freeze-dried cultures of *L. casei* K9-1 and *L. fermentum* K9-2 with a viable cell count of 10^9 CFU / g were provided by CanBiocin Inc. (Edmonton, AB, Canada). The freeze-dried cultures were stored at 4 ºC until use.

4.2.2. Experimental diet preparation

Feed fermentations were performed as previously described [216]. To confirm the identity of the inoculum with fermentation microbiota, the pH, the viable cell counts and the colony morphology of isolates from of each batch of fermented feed were monitored. Viable cell counts were determined by surface plating of serially diluted samples onto MRS5 agar.

The phase I and II basal diets were fed sequentially in the 3-week pig trial and met recommended nutrient requirements for weaning piglets (Table S4.1 of the online supplementary material). The phase I basal diet was fed from day 1 to day 8 and the phase II basal diet from day 9 to 22 (Figure S4.1). The basal diet was mixed with wheat, fermented feeds or probiotic cultures to obtain the following dietary treatments: **Diet A**, unfermented wheat; **Diet B**, unfermented wheat acidified to pH 3.8 with lactic acid and acetic acid; **Diet C**, unfermented wheat with freeze-dried cultures of *L. casei* K9-1 and *L. fermentum* K9-2; **Diet D**, wheat fermented with *L. casei* K9-1 and *L. fermentum* K9-2; **Diet E**, wheat fermented with *L. reuteri* TMW1.656; **Diet F**, wheat fermented *L. reuteri* TMW1.656Δ*rtcN*. Cell counts of the strains *L. casei* K9-1, *L. fermentum* K9-2, *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN* in feed are shown in Table 4.1. The average cell counts of *L. casei* K9-1 supplied as freeze-dried culture (Diet C) or by feed fermentation (Diet D) were 7.46 and 8.08 log (CFU/g), respectively; the cell counts of *L. fermentum* K9-2 supplied in Diet C and

D were 7.25 and 7.68 log (CFU/g), respectively. The estimated daily intake of individual probiotic strains was about 10^{10} -10^{11} CFU / day.

Table 4.1. Viable cell counts of probiotic strains in pig diets

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

[a] Cell counts of respective strains.

[b] Data is represented as mean ± SD. The average was calculated on 25 samples for each diet collected daily over the 3-week animal trial.

[c] n.d., cell counts below the detection limit of 10^5 CFU/g.

4.2.3. Animal experimentation

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

(28 ± 2.5ºC). The six dietary treatments were randomly allocated to 48 piglets in a randomized block design. Each animal was housed in a single pen to provide 8 replicates per dietary treatment. The six experimental diets were administered for 21 days and pigs were killed on day 23. Pigs had free access to feed and water during the trial. Diets were provided at equal amounts twice per day. For bacterial analysis, fresh feces was collected from the pen floors days 0, 7, 14 and 21. The fecal samples were kept at -20ºC upon. Digesta of stomach, ileum, cecum and colon were collected at euthanasia and stored at -20ºC. Frozen samples were thawed, mixed aseptically by spatula and 2-3 g subsamples were stored at -80ºC.

4.2.4. Extraction of DNA from intestinal and fecal microbiota samples

DNA was extracted from intestinal and fecal samples using QIAamp Fast DNA stool mini kit (Qiagen, Inc., Valencia, CA, USA). In brief, approximately 0.2 g of sample was placed into a 2 mL tube filled with 0.2 g of silica beads. The sample was homogenized mechanically followed by heating for 15min at 95ºC. DNA extraction from pure cultures was performed using Blood & Tissue Kit (Qiagen, Inc., Valencia, CA, USA).

4.2.5. Design of strain-specific primers

Strain-specific primers for *L. casei* K9-1 and *L. fermentum* K9-2 were identified by comparative genomic analysis. Genome sequencing, assembly, and annotation of *L. casei* K9-1 and *L. fermentum* K9-2 was conducted by Fusion Genomics (Burnaby, BC, Canada) using *L. casei* ATCC 393 and *L. fermentum* 3872 as reference genomes, respectively. Genome sequences for *L. casei* K9-1 and *L. fermentum* K9-2 were compared to 33 and 19 closed genomes of *L. casei* and *L. fermentum*, respectively (Table S4.2). Strain specific sequences (Table 4.2) were identified by alignment of all genomes of the same species using MAUVE [229] (Figure S4.2, Table S4.2).

Strain-specific primers for *L. reuteri* strains targeted the non-ribosomal peptide synthase RtcN, which is exclusive to five strains of *L. reuteri* including *L. reuteri* TMW1.656 (Table 4.2, Lin et al., 2015). *L. reuteri* TMW1.656Δ*rtcN* was detected with primer targeting the deleted region of *rtcN*. The primer binding sites are also present in the wild-type strain, however, elongation times prevented amplification of the 3047 bp *rtcN* from the wild-type *L. reuteri* TMW1.656. To evaluate primer specificity *in silico*, primers were analysed by BLAST against the nucleotide collection available on Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR reactions indicated that the strain-specific primers resulted in positive amplicons from genomic DNA of the respective strains (Online supplementary Table 4.2).

4.2.6. *In silico* validation of group specific primers

Group-specific primers for *L. reuteri* group, *L. delbrueckii* group, and *L. salivarius* group are shown in Table 4.2. Their specificity was verified with the probe match tool of the Ribosomal Database Project (https://rdp.cme.msu.edu/). Group-specific primers were tested in PCR reactions with template DNA as follows: *L. plantarum* FUA3099, *L. fermentum* K9-2, *L. paralimentarius* FUA3121, *L. sanfranciscensis* FUA3458 and *L. casei* K9-1 were used for validation of the general LAB primers; *L. ruminis* FUA3179, *L. animalis* FUA3045 were used for validation of *L. salivarius* group primers; *L. reuteri* TMW1.656 and *L. vaginalis* FUA3049 were used for validation of *L. reuteri* group primers; *L. crispatus* DSM29598 was used for validation of *L. delbrueckii* group primers.

Table 4.2. Primers used in PCR amplification

Target	Primer	Sequence (5'-3')	Product size (bp)	Tm (ºC)	Reference
Lactobacillus complex[a]	Lab F/ R	AGCAGTAGGGAATCTTCCA / CACCGCTACACATGGAG	341	63	[230]
L. reuteri group	sg-Lreu F/ R	GAACGCAYTGGCCCAA / TCCATTGTGGCCGATCAGT	289	60	[231]
L. delbrueckii group	sg-Ldel F/R	GATGCATAGCCGAGTTGAGAGACTG AT / TAAAGGCCAGTTACTACCTCTATCC	197	60	[231]
L. salivarius group	sg-Lsal F/R	CACCGAATGCTTGCAYTCACC / GCCGCGGGTCCATCCAAAA	182	60	[231]
L. casei K9-1	K9-1F/R	GTTGGAGGATCGCGGATTAG / CGTCACCGGAAGTGATGTT	98	62	This study
L. fermentum K9-2	K9-2F/R	CCCACGAGATTGCCCATATT / GAAGATCCATTGCCGTTTCATTAG	111	62	This study
L. reuteri TMW1.656	WT F/R	ACCGGAACATAACAACACCTTA / GAGGTTCCACCGTCATCAAA	105	62	This study
L. reuteri TMW1.656△*rtcN*	rtcN F/R	ACGTTCTAGTAACACAAGTTGGA / TGTAGAGTGTGCTTGAGGAAAG	134	62	This study

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

4.2.7. Quantitative PCR for detection of probiotic strains

Quantitative PCR reactions were conducted on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Standard curves for qPCR were generated with PCR amplicons obtained with the same primers and genomic DNA of the respective strains as template. Six 10-fold serially diluted standard samples were used as template. The number of gene copies for each standard was calculated based on DNA concentrations as determined using Nano-drop spectrophotometer system (Thermo Fisher Scientific Inc., Wilmington, USA) and the molecular weight of the PCR product. The detection limit of the assay was 10^5 gene copies per g. The qPCR reaction mixture with a total volume of 25 µL contains 12.5 µL of Quanti Fast SYBR Green master mix (Applied Biosystems), 0.5 µL of 10 µM forward/ reverse primers, 1 µL of template DNA and 10.5 µL of RNase-free water. Technical repeats were conducted for all qPCR reactions.

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

HRM-qPCR was conducted on Rotor-GeneQ (Qiagen, USA) using the Type-it HRM PCR Kit (Qiagen, USA) [232] with group specific primers (Table 4.2). Purified 16S rDNA amplicon derived from *L. ruminis* FUA3179, *L. reuteri* TMW1.656 and *L. crispatus* DSM29598 were used as standards in quantification of *L. salivarius* group, *L. reuteri* group, *L. delbrueckii* group, respectively [232]. Multiple species per PCR reaction were identified by cloning of PCR products and Sanger sequencing. In brief, 16S rDNA regions were amplified with group-specific primers followed by purification of the PCR products. The resultant purified 16S rDNA amplicon and the vector pUC19 were ligated by T4 ligase after digestion with *SmaI*. The ligated plasmid was transformed into *E. coli* DH5α followed by plating onto LB agar containing IPTG (0.2 mM), X-gal (40 mg/L) and Ampicillin (50 mg/L).

4.2.9. High throughput sequencing of 16S rRNA sequence tags

Sequences of 16S rRNA sequence tags were obtained on an Illumina MiSeq by the University of Minnesota Genomics Center (Minneapolis, MN, USA). The V5-V6 domain of the 16S rRNA gene was amplified using forward and reverse primers GTGCCAGCMGCCGCGGTAA and CGACRRCCATGCANCACCT, respectively, and the amplicons were pooled for pair-end 300-bp reads sequencing. Sequences were analyzed using the QIIME pipeline (MacQIIME 1.9.1-20150604) [233] After quality control, a total of 6,647,893 sequences with an average length of 266 bp were obtained, corresponding to an average of 34,805 sequences per sample. Operational Taxonomic Units (OTU) clustering was conducted by UCLUST [234] using the GreenGenes database with 97% similarity threshold after quality-filtering and de-multiplexing. Low abundance OTUs with relative abundance < 0.005% of the total OTUs were discarded [235]. The OTU table was filtered by filter_taxa_from_otu_table.py to obtain the OTUs clustered into *Lactobacillus*. The assignment of selected OTUs to phylogenetic groups in the genus *Lactobacillus* [217] was based on BLAST analysis with the sequences in the NCBI database. The relative abundance was calculated as percentage of the abundance of amplicons representing specific bacterial taxa relative to the total abundance of bacterial rDNA. Mixed Procedure based on repeated measurement under randomized block design was applied to the normalized relative abundance of each *Lactobacillus* group.

4.2.10. Statistical analysis

Analysis of qPCR results was performed in SigmaPlot (Systat Software, San Jose, CA). Gene copy numbers of administered probiotic strains, indigenous *L. reuteri* group, *L. delbrueckii* group and *L. salivarius* group in intestinal contents were compared among dietary treatment using two-way

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

4.3. Results

4.3.1. Strain-specific detection of probiotic strains.

A strain-specific quantitative PCR assay was established to monitor the fate of probiotic strains during intestinal transit. Strain-specific primers for *L. reuteri* TMW1.656 and TMW1.656Δ*rtcN* target the reutericyclin biosynthesis gene cluster which is unique to 5 strains of *L. reuteri* [23,225]. The strain-specific primers for *L. casei* K9-1 and *L. fermentum* K9-2 target unique sequences that were identified by comparative genomic analysis (Figure S4.2). Strains were detected in samples from pigs fed the corresponding strains, but not in pigs that were fed other strains, or animals that did not receive probiotics (Table 4.3). The unexpected presence of probiotic strains in few fecal samples of probiotic-free groups (Table 4.3) likely relates to cross-contamination during sampling or DNA handling. Strains were not observed in intestinal samples of animals that did not receive the respective strain in the diet; this observation excludes contamination during feed preparation.

4.3.2. Fate of ingested probiotic strains through piglet GIT

To assess survival of strains with different lifestyles, probiotics were quantified with strain-specific primers in digesta obtained from the stomach, the ileum, the caecum, and the colon (Figure 4.1) and in fecal samples (Table 4.3). Gene copy numbers in colonic digesta were lower ($P < 0.05$) than gene copy numbers in stomach or ileal digesta for all strains except *L. reuteri* TMW1.656Δ*rtcN* (Figure 4.1). The decline in the number of *L. fermentum* K9-2 from proximal GIT (stomach) to distal GIT (colon) was largest relative to other strains. Gene copy numbers of *L. fermentum* K9-2 in the cecum were lower ($P < 0.05$) when delivered as freeze-dried form compared to delivery of the same strain in fermented feed (Figure 4.1). *L. reuteri* wild-type strain had a higher ($P < 0.05$)

gene copy number in the stomach than its reutericyclin-negative isogenic mutant (Figure 4,1). Conforming to the abundance of strain specific DNA in intestinal samples, higher gene copies of the *L. reuteri* strains were detected in fecal samples when compared to *L. casei* K9-1 and *L. fermentum* K9-2 (Table 4.4). Overall, the strain specific detection of lactobacilli in intestinal and fecal samples indicated that the survival of vertebrate host-adapted lactobacilli is higher when compared to other lactobacilli.

Table 4.3. Gene copy number of the orally administered probiotic strains in fecal samples. Data are presented as average ± SD of 8 pigs per diet

Microorganism and time (day)[a]	Log (gene copy#/g of wet feces) for the following diets[b]					
	Control	Acidified controls	*L. casei* / *L. fermentum* freeze-dried	*L. casei* / *L. fermentum* fermented	*L. reuteri* TMW1.656	*L. reuteri* TMW1.656Δ*rtcN*
Lactobacillus complex						
0	9.2±0.9	9.2±0.7	9.4±0.9	9.8±0.4	9.7±0.9	9.3±0.8
7	10.0±0.3	10.3±0.6	10.3±0.6	10.5±0.7	9.9±0.5	9.7±0.5
14	10.0±0.6	9.9±0.8	10.3±0.3	10.0±0.7	9.6±0.6	10.0±0.7
21	9.7±0.8	9.6±0.5	9.7±0.6	10.3±0.7	9.6±0.6	9.5±0.6
L. reuteri group						
0	8.7±1.4	8.1±1.2	8.8±1.8	9.2±1.2	8.6±1.6	9.4±1.4
7	10.1±0.5	10.0±0.8	10.4±0.2	9.6±0.7	9.8±1.3	9.4±1.5
14	10.2±0.4	10.4±0.4	10.3±0.4	10.1±0.5	9.8±0.6	9.7±0.8
21	10.2±0.5	9.9±0.4	10.0±0.3	10.3±0.5	9.5±0.5	9.6±0.7
L. casei K9-1						
0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
7	<5.0	<5.0	8.8±0.3	8.5±0.5	<5.0	<5.0
14	<5.0	<5.0	9.4±0.7^{A}	8.7±0.7^{B}	<5.0	<5.0
21	<5.0	<5.0	9.0±0.4	8.8±0.32	<5.0	<5.0
L. fermentum K9-2						
0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
7	<5.0	<5.0	5.9±0.7^{B}	6.8±1.1^{A}	<5.0	<5.0
14	<5.0	<5.0	6.4±0.6^{A}	6.7±0.5^{A}	5.6±0.6^{B}	<5.0
21	<5.0	<5.0	6.0±0.8	6.8±0.5	<5.0	<5.0
L. reuteri TMW1.656						
0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0
7	<6.0	<6.0	<6.0	<6.0	9.8±0.7	<6.0
14	<6.0	<6.0	<6.0	<6.0	9.4±0.4	<6.0
21	<6.0	<6.0	<6.0	<6.0	9.3±0.2	<6.0
L. reuteri TMW1.656⊿*rtcN*						
0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0
7	<6.0	<6.0	<6.0	7.2±0.8^{B}	<6.0	9.2±0.5^{A}
14	<6.0	<6.0	<6.0	<6.0	<6.0	8.9±0.7
21	<6.0	<6.0	<6.0	<6.0	<6.0	8.9±0.3

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

[b] Gene copy number of respective probiotic strains was determined by qPCR assay. Data are represented as mean ± standard deviations of 8 animals. Data for the same strain in the same row without a common capital superscript differ ($P < 0.05$).

Figure 4.1. Quantification of probiotic lactobacilli in stomach, ileal, cecal, and colonic digesta. Animals received diets containing *L. casei* K9-1 (dark gray bars) and *L. fermentum* K9-2 (light gray bars) in freeze-dried from (**hatched bars**, Diet C), or through fermentation (Diet D), *L. reuteri* TMW1.656 (Diet E) or *L. reuteri* TMW1.656Δ*rtcN* (white bars, Diet F). Digesta were sampled at sacrifice after 3 weeks of feeding. Probiotic organisms were detected with strain specific primers to quantify *L. casei* K9-1 (**dark gray bars**); *L. fermentum* K9-2 (**light gray bars**); *L. reuteri* TMW1.656 (**black bars**), and *L. reuteri* TMW1.656Δ*rtcN* (**white bars**). Data are presented as mean (n=8) ± standard deviations of 8 replicate observations. One-way ANOVA was performed to assess differences of gene copy numbers between different strains in the same region of gut. Gene copy numbers of different strain in the same compartment of the intestine are significantly ($P < 0.05$) different if bars do not share a common lower case superscript. Gene copy numbers of strains in colonic digesta are marked with an asterisk if they were lower ($P < 0.05$) in comparison to gene copy numbers of the same strain in stomach digesta.

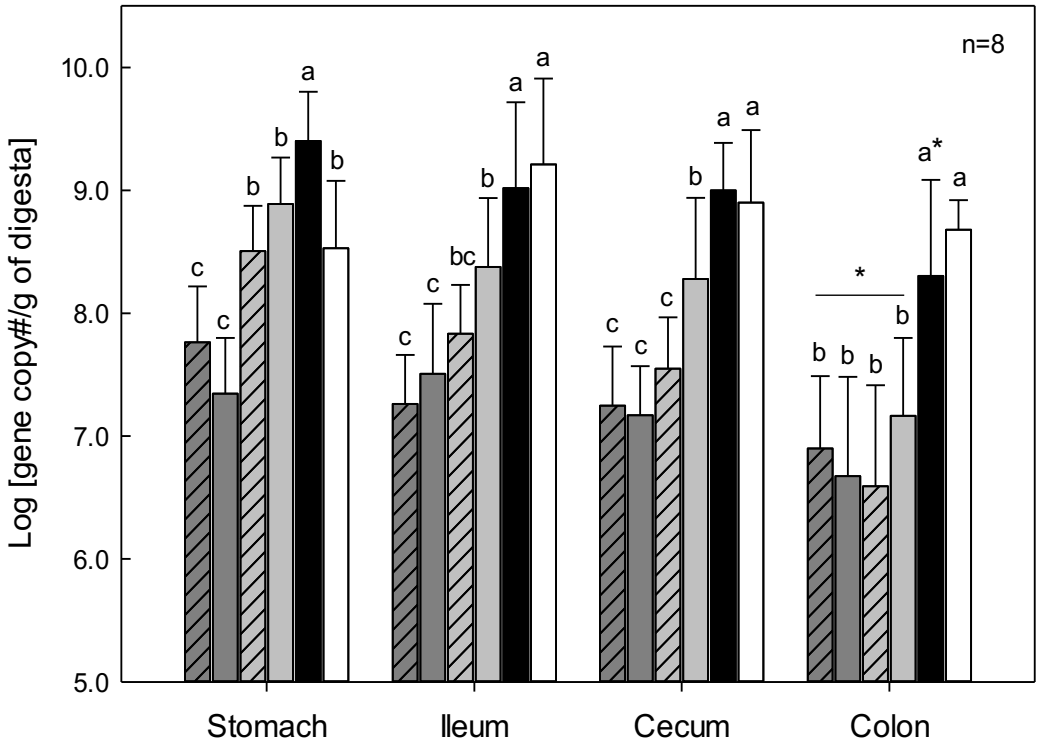


Table 4.4. Abundance of rDNA corresponding to the *L. reuteri* group, *L. salivarius* group, *L. delbrueckii* group and *Lactobacillus* spp. relative to total bacterial rDNA in feces of piglets during the first 3 weeks post weaning. Data were determined by sequencing of 16S rRNA tags and are represented as mean ± SD of 8 pigs. Within each row, means without common capital superscript differ ($P < 0.05$). Within each column, means without common lowercase superscript differ ($P < 0.05$).

Group / Collection day	Control	Acidified controls	*L. casei* / *L. fermentum* freeze-dried	*L. casei* / *L. fermentum* fermented	*L. reuteri* TMW1.656	*L. reuteri* TMW1.656Δ*rtcN*
L. reuteri group						
0	0.6±0.7 $^{B, b}$	2.1±3.2 ^{A}	1.7±2.0 $^{AB, b}$	0.7±1.0 $^{B, b}$	1.7±2.4 ^{AB}	0.6±0.76 $^{B, b}$
7	1.7±1.2 $^{AB, a}$	3.7±3.9 ^{AB}	2.5±1.6 $^{AB, ab}$	4.7±4.0 $^{A, a}$	1.6±1.3 ^{B}	2.1±2.29 $^{AB, ab}$
14	3.4±3.3 $^{A, a}$	2.7±1.8 ^{AB}	3.6±2.1 $^{A, a}$	2.3±1.2 $^{AB, a}$	1.1±0.5 ^{B}	2.1±1.68 $^{AB, a}$
21	1.6±0.6 $^{AB, a}$	2.5±2.4 ^{AB}	2.1±1.2 $^{AB, ab}$	2.7±1.4 $^{A, a}$	1.0±0.3 ^{B}	2.7±2.47 $^{AB, a}$
L. salivarius group						
0	0.05±0.06	0.1±0.2	0.04±0.06	0.03±0.1	0.08±0.1	0.02±0.04
7	0.03±0.05 ^{AB}	0.03±0.07 ^{AB}	0.08±0.1 ^{AB}	1.7±4.7 ^{A}	0.08±0.1 ^{AB}	0.01±0.02 ^{B}
14	0.02±0.02	0.02±0.03	0.03±0.07	0.03±0.04	0.01±0.01	0.01±0.02
21	0.05±0.10	0.03±0.07	0.01±0.01	0.04±0.05	0.00±0.00	0.01±0.01
L. delbrueckii group						
0	1.9±1.5 $^{B, b}$	2.9±3.3 $^{AB, b}$	3.0±2.6 $^{AB, b}$	1.9±1.7 $^{B, b}$	4.0±4.1 ^{A}	2.5±3.6 $^{AB, b}$
7	6.8±6.5 ^{a}	11.5±7.3 ^{a}	7.4±4.8 ^{a}	12.6±13.7 ^{a}	6.2±4.6	9.1±12.2 ^{a}
14	4.6±2.9 ^{a}	5.6±4.6 ^{ab}	6.0±2.7 ^{a}	5.9±4.1 ^{a}	4.2±2.0	9.1±6.7 ^{a}
21	5.5±3.5 ^{a}	8.8±4.0 ^{a}	5.3±2.0 ^{a}	6.0±3.3 ^{a}	7.1±3.7	6.8±3.7 ^{a}
Lactobacillus spp.						
0	2.5±1.6 $^{B, b}$	5.1±5.8 $^{A, b}$	4.8±4.4 $^{A, b}$	2.7±2.5 $^{B, b}$	5.8±6.2 ^{A}	3.1±4.3 $^{AB, b}$
7	8.5±7.5 $^{B, a}$	15.2±6.3 $^{A, a}$	10.0±5.0 $^{AB, a}$	19.1±20.5 $^{A, a}$	7.9±5.0 ^{B}	11.2±14.3 $^{AB, a}$
14	8.0±3.5 $^{AB, a}$	8.3±4.9 $^{AB, ab}$	9.6±3.7 $^{A, a}$	8.3±4.8 $^{AB, a}$	5.1±2.5 ^{B}	11.2±7.1 $^{A, a}$
21	7.1±3.6 ^{a}	11.30±5.31 ^{a}	7.5±2.5 ^{a}	8.7±3.1 ^{a}	8.0±3.8	9.6±5.4 ^{a}

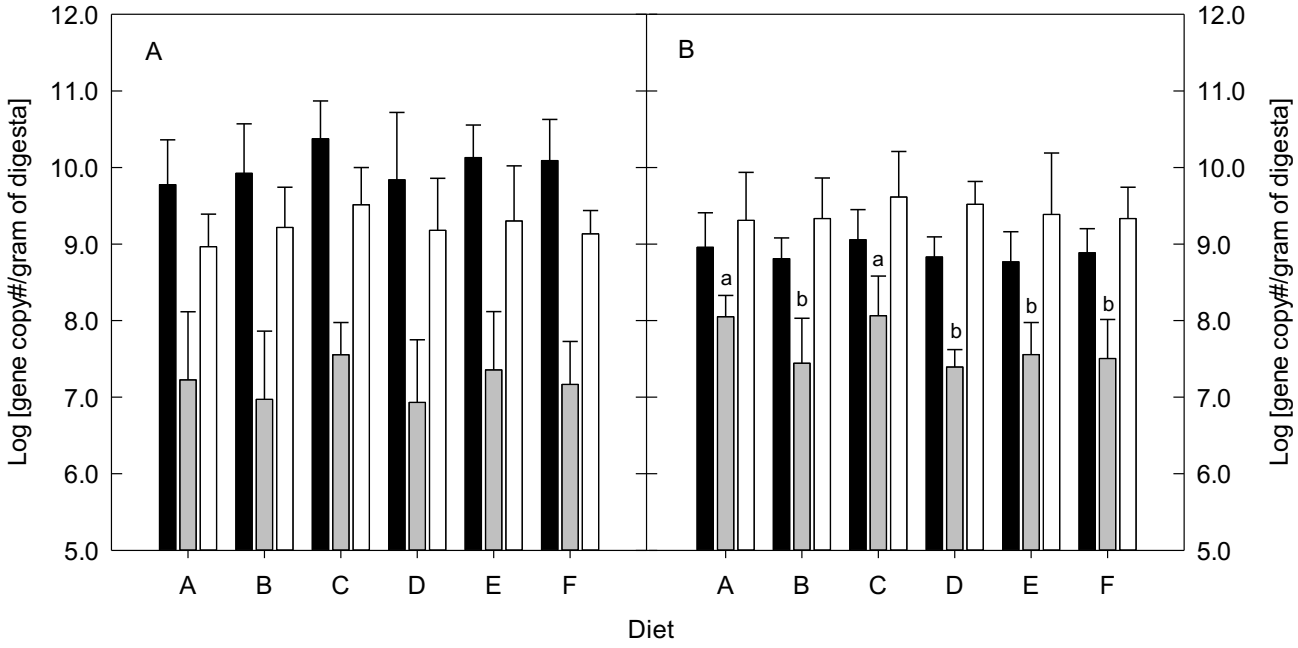
4.3.3. Effect of probiotic strains on abundance and composition of autochthonous lactobacilli

To characterize the impact of probiotics on autochthonous lactobacilli, the abundance and composition of autochthonous lactobacilli was analyzed in digesta samples collected at the end of the trial. Intestinal lactobacilli all belonged to the *L. reuteri* group, the *L. delbrueckii* group, or the *L. salivarius* group (Table 4.2). Group-specific HRM-qPCR quantified representatives of these *Lactobacillus* groups. *L. reuteri* constituted the most abundant group in the stomach; the *L. delbrueckii* group was most abundant in colonic digesta while the *L. salivarius* group was a minor component in both intestinal compartments (Figure 4.2). Probiotics did not alter the composition of *Lactobacillus* populations in the stomach (Figure 4.2A). The abundance of the *L. salivarius* group in the colon was decreased ($P < 0.05$) in animals fed chemically acidified feed or fermented feed, indicating that organic acids may contribute to this effect (Figure 4.2B).

HRM-qPCR discriminates between 16S rDNA amplicons obtained with the same primers by analysis of the melting temperature (Tm), and thus discriminates between closely related species which differ with respect to the Tm of amplicons. Melting peaks obtained in HRM-qPCR analysis were assigned to specific *Lactobacillus* species by using reference strains, Sanger sequencing of PCR amplicons, or 16S rRNA sequences from fecal samples of same piglet. Two melting peaks with Tm 81.8°C and 82.9°C were consistently observed after amplification of 16S rDNA from the *L. salivarius* group in colonic digesta of 10 piglets from all six dietary treatments. Only one melting peak with a Tm of 81.8°C was observed in samples of remaining 38 piglets (Figure S4.3A). Sanger

sequencing of PCR amplicons from one of the piglets identified amplicons with Tm of 81.8ºC and 82.9ºC as amplicons from *L. salivarius* and *L. ruminis*, respectively (Figure S4.3A). This assignment matched the predicted Tm of 16S rDNA from *L. ruminis* and *L. salivarius*. *L. salivarius* accounted for about 99% of *L. salivarius* groups organisms in fecal samples (Table 4.4 and data not shown), in agreement with the consistent presence of the amplicon with Tm 81.8ºC in intestinal samples from all piglets (Figure S4.3A). In colonic digesta, a single melting peak at Tm 84.1ºC was observed with *L. reuteri* group specific primers (Figure S4.3B). This Tm matches the Tm of the reference strain of *L. reuteri,* and the prevalence of OTUs assigned to *L. reuteri* in 16S sequences of fecal samples. A single peak at Tm of 84.5ºC was observed in all samples with primers specific for the *L. delbrueckii* group (Figure S4.3C). This Tm matches the predicted Tm of *L. amylovorus* (84.75°C), *L. johnsonii* (85.0°C), as well as *L. gasseri* (84.75°C); these species can thus not be differentiated by the HRM-qPCR as used in this study. OTUs assigned to *L. amylovorus* and (*L. gasseri* or *L. johnsonii*) accounted for more than 97% of all sequences assigned to the *L. delbrueckii* group in fecal samples. In short, HRM-qPCR of intestinal samples provided no indication that probiotic feeding influenced the composition of intestinal lactobacilli at the species level.

Figure 4.2. Gene copy numbers of *L. reuteri* group (black bar), *L. salivarius* group (gray bar), *L. delbrueckii* group (white bar) in digesta obtained from the stomach (Panel A) and the colon (Panel B) of piglets. Digesta were sampled at sacrifice after 3 weeks of feeding. The capital letter codes at X-axis indicate respective diet: Diet A, control; Diet B, acidified control; Diet C, *L. casei* K9-1 and *L. fermentum* K9-2 in freeze-dried form; Diet D, *L. casei* K9-1 and *L. fermentum* K9-2 in fermented wheat; Diet E, *L. reuteri* TMW1.656 in fermented wheat; Diet F, *L. reuteri* TMW1.656Δ*rtcN* in fermented wheat. Data is expressed as mean ± standard deviation of 8 individual piglets. The gene copy number was quantified using HRM-qPCR method with a detection limit of 1×10^5 gene copies/ g of digesta. Significant differences between gene copy numbers from animals fed different diets were assessed by one-way ANOVA. Bars without a common superscript differ ($P < 0.05$); superscripts are not shown if none of the values were different.

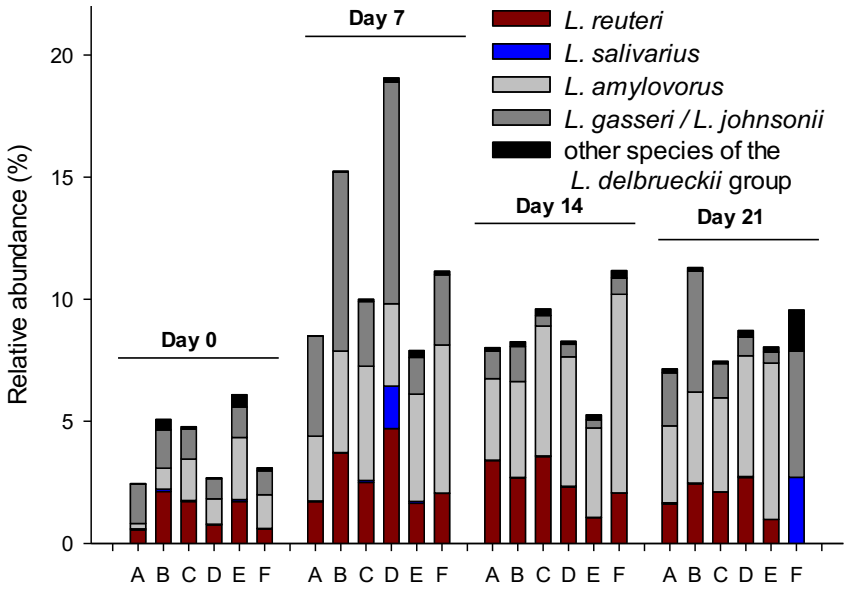


4.3.4. Effect of probiotic strains on autochthonous *Lactobacillus* communities in feces

The alteration of intestinal *Lactobacillus* communities in response to probiotics was further analyzed by high throughput sequencing of 16S rRNA sequence tags of PCR amplicons from fecal samples. In keeping with prior observations with *L. reuteri* TMW1.656 in weanling piglets [23]

probiotic feeding did not induce major change for total fecal microbiota (Figure S4.4). Sequences matching to the genus *Lactobacillus* were initially analyzed at the level of the phylogenetic group [217]; results are shown in Table 4.3. Identification of most lactobacilli at the species level was achieved by a combination of 16S rRNA sequence data and the species-level identification with HRM-qPCR; results of the species level identification are shown in Figure 4.3. Individual animals differed substantially with respect to the *Lactobacillus* microbiota at baseline (Table 4.4). The abundances of lactobacilli and the three major *Lactobacillus* groups fluctuated in the first weeks but stabilized after week 2 (Table 4.4 and Figure 4.3). Probiotic interventions with *L. casei* and *L. fermentum* did not affect the abundance of *L. reuteri* group, *L. delbrueckii* group or *L. salivarius* group (Table 4.4). Administration of the reutericyclin-positive *L. reuteri* TMW1.656 transiently decreased the abundance of the *L. reuteri* group in fecal samples in comparison to control and *L. casei* / *L. fermentum* fed animals (Table 4.4) and reduced the proportion of lactobacilli in comparison to the reutericyclin-negative mutant (Figure 4.3). These results suggest that reutericyclin is a subtle but significant modulator of the *Lactobacillus* community in pigs.

Figure 4.3. Relative abundance of the species *L. reuteri*, *L. salivarius*, *L. amylovorans*, *L. gasseri* / *L. johnsonii*, and other members of the *L. delbrueckii* group in feces of pigs during the first three weeks post weaning. Sequencing of 16S rRNA tags allowed assignment of *Lactobacillus* sequences at the level of the phylogenetic group (Table 4.4); the assignment of sequences to specific *Lactobacillus* species was enabled by combination of rDNA sequence data with HRM-qPCR and Sanger sequencing of PCR amplicons (Figure S3). Bars indicate the average abundance of *Lactobacillus* species relative to total rDNA. Different colors represent different species as indicated; letters indicate the different diet as follows. Diet A: control, Diet B: acidified control, Diet C: *L. casei* K9-1 and *L. fermentum* K9-2 in freeze-dried form, Diet D: *L. casei* K9-1 and *L. fermentum* K9-2 in fermented form, Diet E: *L. reuteri* TWM1.656, Diet F: *L. reuteri* TMW1.656 Δ*rtcN*. Data without a common capital superscript differ ($P < 0.05$).



4.4. Discussion

The present study investigated the interaction between probiotic bacteria of non-swine origin and *Lactobacillus* communities in the pig intestine. We employed probiotic *L. fermentum*, *L. casei*, and *L. reuteri* strains in weaned piglets to i) compare the effect of freeze-dried culture versus fermented cultures on probiotic efficacy in the piglet GIT; ii) develop a culture-independent method for specific quantification of probiotic strains during intestinal transit; and iii) to explore the *in vivo* ecological role of reutericyclin production by *L. reuteri*. The absolute amount and relative abundance of three indigenous *Lactobacillus* groups in various regions of the gut, i.e. stomach, ileum, cecum and colon, and in feces, were detected using strain-, group-specific HRM-qPCR, and 16S rDNA amplicon sequencing.

4.4.1. Development of a strain-specific qPCR assay to enumerate probiotic strains

Strain-specific quantitative PCR differentiated probiotic strains from autochthonous lactobacilli throughout the intestine of weaned piglets. The availability of genome sequences enables identification of strain-specific primers by comparative genomics. Previously, typing methods, such as Random Amplification of Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP) and Suppression Subtractive Hybridization (SSH) were used to distinguish between bacterial isolates [236–245]. In addition, strain specific qPCR primers were designed based on strain-specific RAPD banding patterns [246,247] unique metabolic traits [248] or ITS-sequences [249]. Studies employing these assays are summarized in Table S4.3. Limitations of these assays include the limited specificity and the

requirement for bacterial culture. For example, RAPD and AFLP analyses require re-isolation of strains and cover only 1 - 10% of a bacterial genome, which may be insufficient for strain-level differentiation. SSH relies on the comparison to only one organism [237,250]. The present study thus provides a novel approach for strain-specific quantification of probiotic *L. fermentum*, *L. casei* and *L. reuteri* by qPCR.

4.4.2. Survival of freeze-dried and fresh probiotic cultures

The survival of *L. fermentum* K9-2 in the GI tract was increased when this strain was provided as part of fermented feed compared to delivery of the same strain as freeze-dried culture. In contrast, survival of *L. casei* K9-1 did not depend on the form of delivery. The survival of freeze-dried probiotic strains during intestinal transit is affected by multiple factors including the culture conditions and the pH at harvest of probiotic bacteria, the use of cryoprotectants during freeze-drying, and the composition of the food matrix used for probiotic delivery[251]. In feed applications, endospores of *Bacillus* spp. are currently preferred as probiotic additives due to the resistance of *Bacillus* endospores to the high temperatures during feed production and feed distribution [252,253]. Feed fermentation with probiotic cultures is a viable alternative for delivery of probiotics in animal production that eliminates the need for strain preparations with high resistance to heat and dry storage [227]. By analogy, the use of probiotic strains as starter cultures in food fermentations [254] may improve their survival during intestinal transit.

4.4.3. Lifestyle of lactobacilli relate to intestinal survival

Strain-specific primers provided a powerful tool to analyze survival of probiotic lactobacilli and their interaction with autochthonous lactobacilli. The lifestyle of lactobacilli was proposed to determine their suitability for probiotic applications [131]; however, this claim has not been substantiated experimentally. *L. casei*, *L. fermentum* and *L. reuteri* represent nomadic, free-living and host-adapted organisms, respectively [44]. The host-adapted *L. reuteri* strain survived better in the swine intestine compared to nomadic or free-living species. Our study complements and expands observations in human subjects [241] *L. reuteri* is a symbiont of pigs; the phylogenetic clade IV of *L. reuteri* has evolved separately from other host-specific clades of *L. reuteri* [255]. However, genetic signatures distinguishing pig-derived *L. reuteri* from strains of other host-adapted clades are lacking[222] and clade IV pig isolates do not outcompete other strains of *L. reuteri* in the pig gut [44]. The lack of swine-specific metabolic traits of *L. reuteri* may account for the improved survival of *L. reuteri* TMW1.656, a sourdough isolate of the rodent-specific clade III [217,223] in the pig intestine. The present study thus supports the hypothesis that adaptation of lactobacilli to vertebrate hosts is a relevant criterion for selection of probiotic strains [44,256].

4.4.4. Impact of probiotic strains on autochthonous lactobacilli

Despite colonization resistance of intestinal microbiota, increasing evidence indicates a role of probiotic strains in modulating autochthonous microbiota if strains are adapted to vertebrate hosts, or specifically to the host species. Generally, probiotics have only a limited impact on the resident gut microbiome [257] and probiotic strains are detectable only for a few days after intake of the

probiotic ends [258]. However, temporary or permanent persistence of probiotic strains was observed when the ecological niche was not occupied by closely related species and when the probiotic strain was adapted to the host species [259,260] The present study investigated weaned piglets, which undergo major shifts in intestinal microbiota including intestinal lactobacilli in the first two weeks post-weaning [23,106,138,261]. Feed fermentation and probiotic lactobacilli were subtle yet significant modulators on the population of autochthonous lactobacilli. The abundance of the *L. salivarius* group was decreased by feed that was acidified chemically or by fermentation (Figure 4.2). Strain-specific effects of probiotics on composition of intestinal lactobacilli were observed only for the reutericyclin-producing *L. reuteri* TMW1.656. Production of antimicrobial metabolites by probiotic strains is regarded as an important trait for probiotic functionality; past studies particularly discussed bacteriocin formation as a potential probiotic trait [240,262]. *L. reuteri* TMW1.656, a strain producing the low-molecular weight antimicrobial compound reutericyclin, affected intestinal microbiota of piglets when compared to a reutericyclin-negative wild-type strain of *L. reuteri* [23]. We investigated the role of reutericyclin in shaping *Lactobacillus* populations by comparison of *L. reuteri* TMW1.656 to a reutericyclin-deficient isogenic mutant. The reutericyclin producing *L. reuteri* TWM1.656 persisted better in the stomach of piglets when compared to the reutericyclin-negative mutant; reutericyclin-production by *L. reuteri* also altered fecal *Lactobacillus* communities, indicating that reutericyclin production may displace sensitive autochthonous lactobacilli.

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

Table S4.1. Ingredient composition of basal diets

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

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

Table S4.2. Genomes used for multiple genome alignment

Genome Accession	NCBI FTP site
Lactobacillus casei	
GCA_000014525.1_ASM1452v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/014/525/GCA_000014525.1_ASM1452v1
GCA_000019245.3_ASM1924v3	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/019/245/GCA_000019245.3_ASM1924v3
GCA_000026485.1_ASM2648v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/026/485/GCA_000026485.1_ASM2648v1
GCA_000194765.1_ASM19476v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/194/765/GCA_000194765.1_ASM19476v1
GCA_000194785.1_ASM19478v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/194/785/GCA_000194785.1_ASM19478v1
GCA_000309565.2_ASM30956v2	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/565/GCA_000309565.2_ASM30956v2
GCA_000309585.1_ASM30958v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/585/GCA_000309585.1_ASM30958v1
GCA_000309605.1_ASM30960v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/605/GCA_000309605.1_ASM30960v1
GCA_000309625.1_ASM30962v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/625/GCA_000309625.1_ASM30962v1
GCA_000309645.1_ASM30964v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/645/GCA_000309645.1_ASM30964v1
GCA_000309665.1_ASM30966v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/665/GCA_000309665.1_ASM30966v1
GCA_000309685.1_ASM30968v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/685.GCA_000309685.1_ASM30968v1
GCA_000309705.1_ASM30970v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/705/GCA_000309705.1_ASM30970v1
GCA_000309725.1_ASM30972v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/725/GCA_000309725.1_ASM30972v1
GCA_000309745.1_ASM30974v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/745/GCA_000309745.1_ASM30974v1
GCA_000309765.1_ASM30976v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/765/GCA_000309765.1_ASM30976v1
GCA_000309785.1_ASM30978v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/309/785/GCA_000309785.1_ASM30978v1
GCA_000318035.1_ASM31803v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/318/035/GCA_000318035.1_ASM31803v1
GCA_000376145.1_ASM37614v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/376/145/GCA_000376145.1_ASM37614v1
GCA_000388095.2_LcY_assembly050913	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/388/095/GCA_000388095.2_LcY_assembly050913
GCA_000400585.1_LcA_0213	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/400/585/GCA_000400585.1_LcA_0213
GCA_000418515.1_ASM41851v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/418/515/GCA_000418515.1_ASM41851v1
GCA_000472345.1_ASM47234v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/472/345/GCA_000472345.1_ASM47234v1
GCA_000474615.1_Lcasei5b_2.0	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/474/615/GCA_000474615.1_Lcasei5b_2.0
GCA_000510825.1_ASM51082v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/510/825/GCA_000510825.1_ASM51082v1
GCA_000615205.1_ASM61520v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/615/205/GCA_000615205.1_ASM61520v1
GCA_000736295.3_L._casei_Hybrid_assembly	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/736/295/GCA_000736295.3_L._casei_Hybrid_assembly
GCA_000827145.1_ASM82714v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/827/145/GCA_000827145.1_ASM82714v1
GCA_000829055.1_ASM82905v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/829/055/GCA_000829055.1_ASM82905v1
GCA_001013375.1_ASM101337v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/013/375/GCA_001013375.1_ASM101337v1
GCA_001066565.1_ASM106656v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/066/565/GCA_001066565.1_ASM106656v1
GCA_001066695.1_ASM106669v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/066/695/GCA_001066695.1_ASM106669v1
GCA_001433735.1_ASM143373v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/433/735/GCA_001433735.1_ASM143373v1

Genome Accession	NCBI FTP site
Lactocbacillus fermentum	
GCA_000010145.1_ASM1014v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/010/145/GCA_000010145.1_ASM1014v1
GCA_000159215.1_ASM15921v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/159/215/GCA_000159215.1_ASM15921v1
GCA_000162395.1_ASM16239v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/162/395/GCA_000162395.1_ASM16239v1
GCA_000210515.1_ASM21051v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/210/515/GCA_000210515.1_ASM21051v1
GCA_000397165.1_ASM39716v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/397/165/GCA_000397165.1_ASM39716v1
GCA_000417005.1_ASM41700v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/417/005/GCA_000417005.1_ASM41700v1
GCA_000466785.3_ASM46678v3	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/466/785/GCA_000466785.3_ASM46678v3
GCA_000472265.1_LF1_1.0	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/472/265/GCA_000472265.1_LF1_1.0
GCA_000477515.1_Reference_Assembly	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/477/515/GCA_000477515.1_Reference_Assembly
GCA_000496435.1_LfermNB22_1.0	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/496/435/GCA_000496435.1_LfermNB22_1.0
GCA_000966835.1_ASM96683v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/966/835/GCA_000966835.1_ASM96683v1
GCA_001010185.1_ASM101018v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/010/185/GCA_001010185.1_ASM101018v1
GCA_001010245.1_ASM101024v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/010/245/GCA_001010245.1_ASM101024v1
GCA_001039735.1_LFE2	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/039/735/GCA_001039735.1_LFE2
GCA_001077025.1_ASM107702v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/077/025/GCA_001077025.1_ASM107702v1
GCA_001297025.1_ASM129702v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/297/025/GCA_001297025.1_ASM129702v1
GCA_001297905.1_ASM129790v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/297/905/GCA_001297905.1_ASM129790v1
GCA_001368755.1_LF_newbler2.7	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/368/755/GCA_001368755.1_LF_newbler2.7
GCA_001436835.1_ASM143683v1	ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/436/835/GCA_001436835.1_ASM143683v1

Table S4.3. Summary of **s**tudies reporting strain-specific identification or quantification methods

Strain	Culture independent (Yes/No)	Method	Identification/ quantification	Sample type	Ref.
L. reuteri DSM 16350	Yes	SSH & strain specific qPCR	Quantification	Chicken feed and intestine	(Sattler et al., 2014)
L. sobrius 001	Yes	Representational difference analysis (RDA) & strain-specific qPCR	Quantification	Pure culture mix	(Konstantinov et al., 2005)
L. rhamnosus GG	Yes	RAPD & strain-specific qPCR	Quantification	Human feces	(Ahlroos and Tynkkynen, 2009)
Lactococcus lactis subsp. *cremoris* FC	Yes	RAPD & strain-specific qPCR	Quantification	Human feces	(Maruo et al., 2006)
B. longum Y10, *B. infantis* Y1 and *B. breve* Y8	Yes	ITS (*in silico* comparison) & strain-specific qPCR	Quantification	Yogurt/ Human feces	(Vitali et al., 2003)
L. gasseri 4B2	No	Colony-multiplex PCR	Identification	Mouse feces	(Lucchini et al., 1998)
L. rhamnosus Lc 1/3	Yes	RAPD & PCR	Identification	Pure culture mix	(Tilsala-Timisjärvi and Alatossava, 1998)
L. paracasei LTH 2579	No	Subtraction hybridization & PCR	Quantification	Fermented sausage/ human feces	(Bunte et al., 2000)
L. paracasei IMPC2.1	Yes	f-AFLP & PCR	Identification	Pure culture mix	(Sisto et al., 2009)
L. rhamnosus 35	Yes	Subtractive hybridization & PCR	Identification	Pure culture mix	(Coudeyras et al., 2008)
L. gasseri K7	Yes	qPCR targeting bacteriocin gene	Quantification	Human feces	(Treven et al., 2013)
L. casei strain Shirota	Yes	RAPD & strain-specific qPCR	Quantification	Human feces	(Fujimoto et al., 2008)
L. reuteri TMW1.656	Yes	*in silico* comparison & strain-specific qPCR targeting RTC biosynthesis gene	Quantification	Pig intestine and feces	(Yang et al., 2015a)
Bifidobacterium longum AH1206	Yes	*in silico* comparison & strain-specific qPCR	Quantification	Human feces	(Maldonado-Gómez et al., 2016)
L. salivarius abp118	No	Selective medium for rifampicin resistance plasmid	Quantification	Mouse and pig intestine and feces	(Riboulet-Bisson et al., 2012)
L. reuteri ATCC PTA 6475 and *L. mucosae* FSL-04	No	RAPD typing	Quantification	Human feces	(Frese et al., 2012)
Bifidobacterium breve strain Yakult (BbrY)	No	RAPD & strain-specific qPCR	Quantification	Human feces	(Fujimoto et al., 2011)

Figure S4.1. Overview of the animal experimental design. Animals were started on the experimental diets at day 1 after weaning.

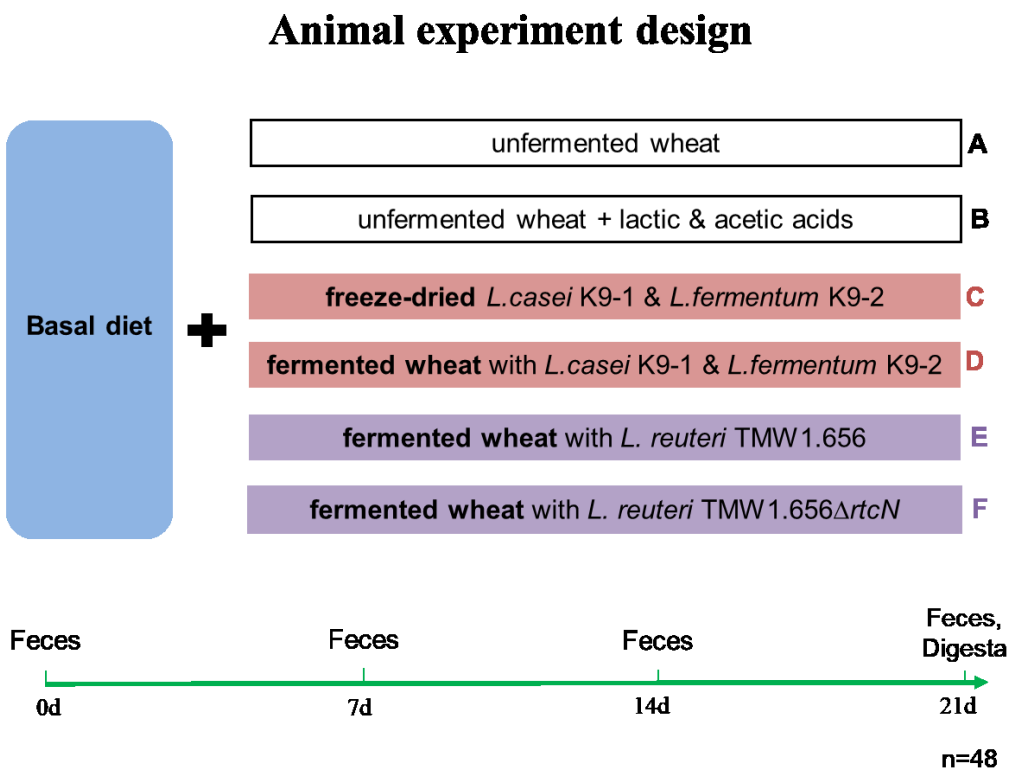
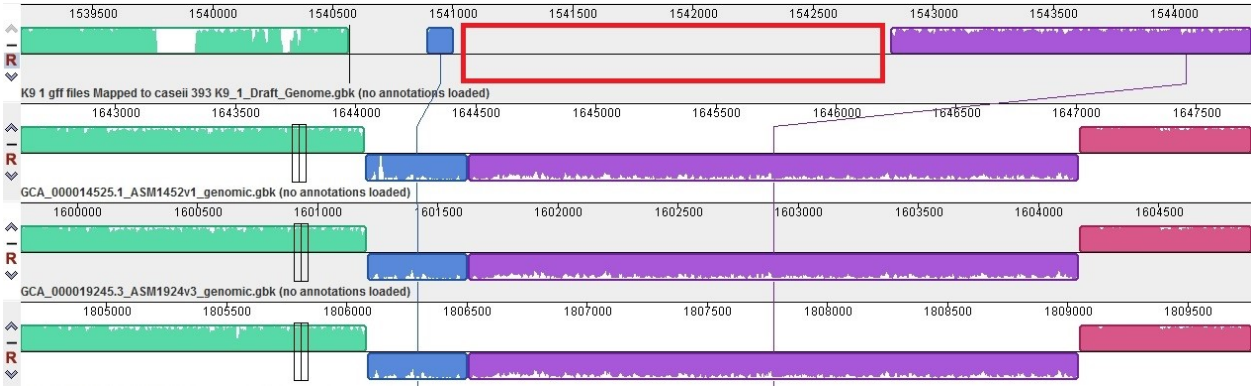


Figure S4.2. Genome alignments of *L. casei* and L. fermentum for design of strain specific primers. **(Panel A)** Genome alignment of *L. casei* K9-1 against genomes of *L. casei*. Shown is the comparison of an area of interest to the three of 33 strains that are most closely related to *L. casei* K9-1. The white area was selected as unique sequence region (highlighted by red box) for strain specific primer design. **(Panel B)** Genome alignment of *L. fermentum* K9-2 against genomes of *L. fermentum*. Shown is the comparison of an area of interest to the two of 19 strains that are most closely related to *L. fermentum* K9-2. The brown block was selected as unique sequence region (highlighted by red box) for strain specific primer design.

(A)



(B)

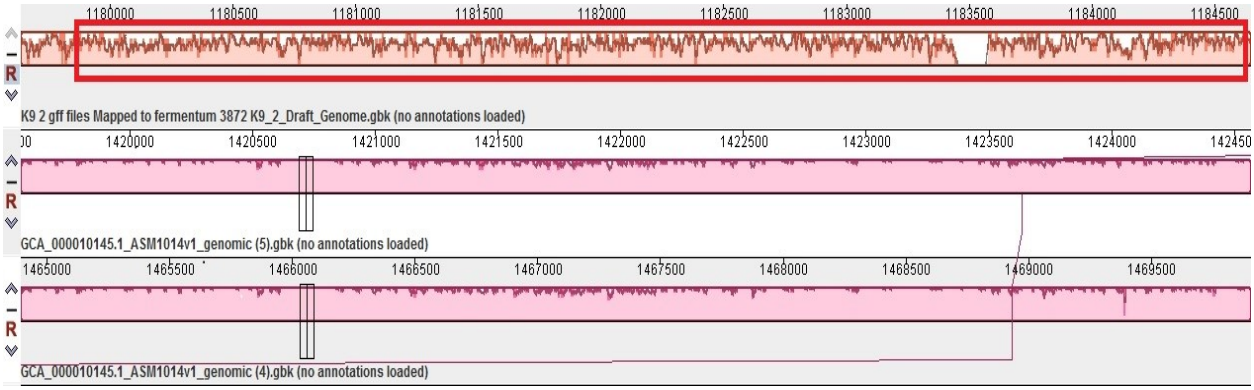


Figure S4.3. First derivatives of melt curves of PCR products obtained from digesta microbiota DNA with *Lactobacillus* group-specific primers. (**Panel A**) *L. salivarius* group primer. Based on the Sanger sequencing and Tm values of reference strains, two peaks shown in above spectrum are presumably assigned to *L. salivarius* (Peak 1 as marked on the curve) and *L. ruminis* (Peak 2 as marked on the curve). OTU's matching to *L. salivarius* was most abundant in 16S sequencing. Colon digesta of Piglet #10 were used for melt curves shown. (**Panel B**) *L. reuteri* group primer. The peak shown in the above spectrum is assigned to *L. reuteri* matching the Tm value of the reference strain. OTUs matching to *L. reuteri* were most abundant in 16S sequencing. Colonic digesta of Piglet #10 were used for the melt curves shown. (**Panel C**) *L. delbrueckii* group primer. The peak shown in above spectrum was assigned to *L. amylovorus* or *L. johnsonii* or *L. gasseri* based on estimated Tm value of respective species and fecal OTU composition. Colonic digesta of Piglet #10 were used for melt curves.

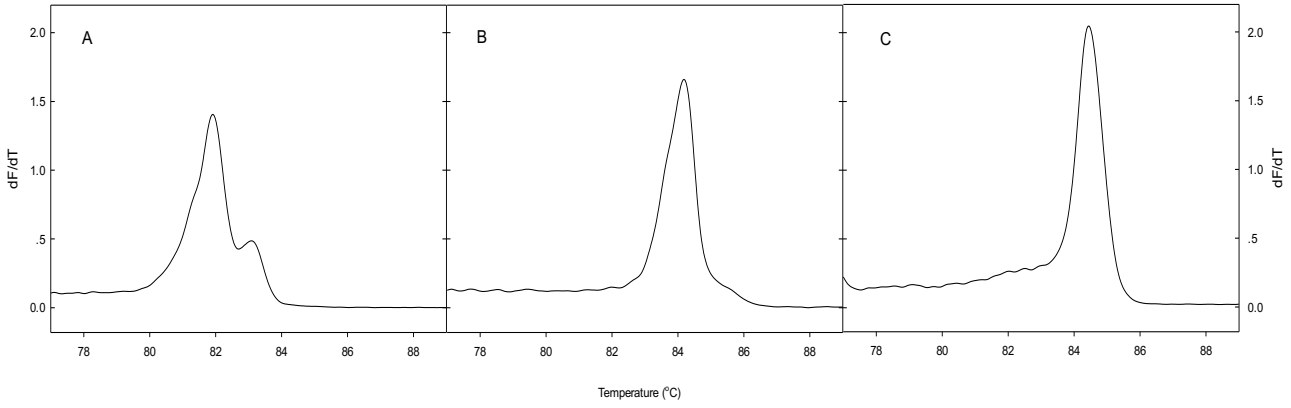
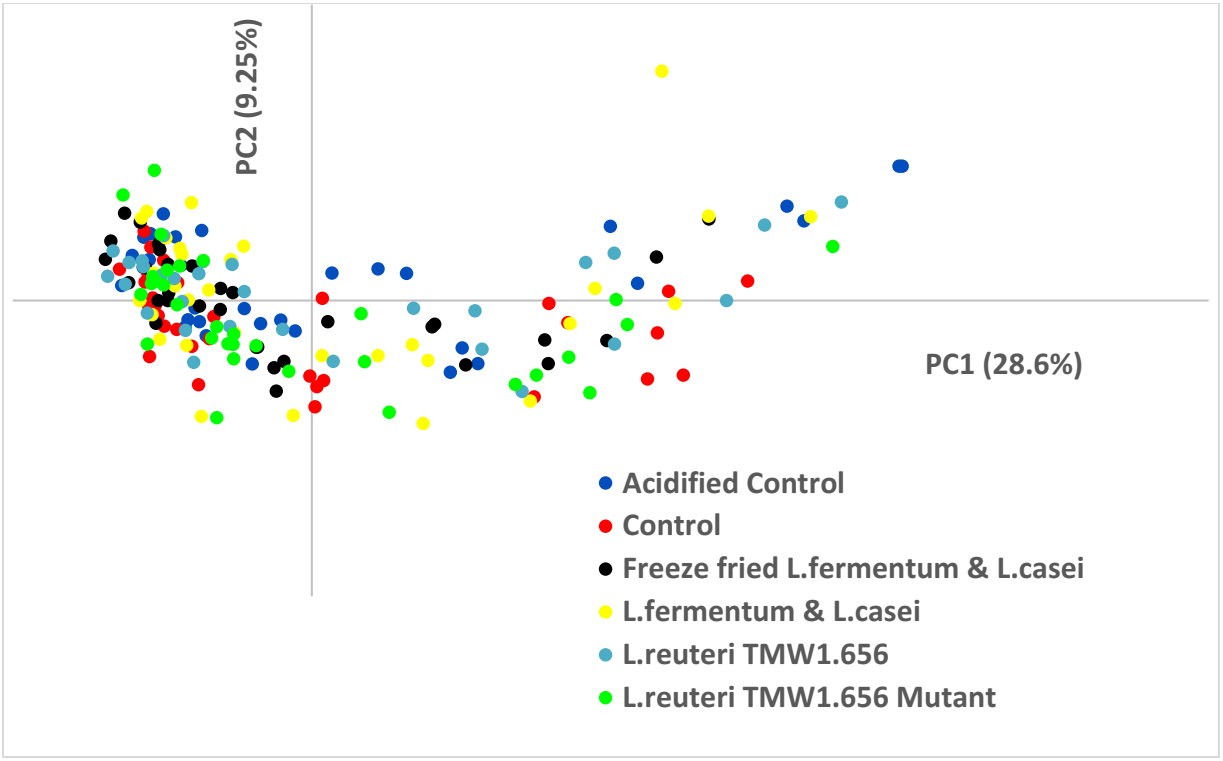


Figure S4.4. Principle Coordinate Analysis (PCoA) of fecal microbiota composition based on unweighted unifrac distance of rDNA sequences (total of 6,647,893 sequences with an average length of 266 bp, corresponding to an average 34,805 sequences per sample). No significant correlation ($P = 0.122$, R = 0.008) between diets and fecal microbial composition was detected by Analysis of Similarities (ANOSIM)



Chapter 5. Feeding *Lactobacillus fermentum*, *Lactobacillus casei*, or *Lactobacillus reuteri* reduces pathogen load in weanling pigs

5.1. Introduction

The stress triggered by weaning and the immature immune system contributed to the high susceptibility of weanling pig to gut microbial dysbiosis and pathogen infection [49,213]. The prevalence of *Clostridoides. perfingens* Type C associated clostridial infections is common in neonatal piglets within 7 days of farrowing and ETEC infection is the most significant cause of post-weaning diarrhea [50,263]. F18 and K88 positive ETEC strains are the most frequently identified cause for ETEC associated diarrheal in nursing and early weaned pigs [264]. In the infant gut, *E. coli* strains could be transmitted from both maternal sources and non-maternal sources. Limited success has been achieved in vaccination against ETEC infectious due to the non-invasive pathogenesis [84,265]. Especially, alternative protection against pathogen infection is required for early weaned pigs due to the interrupted protection from maternal immunity. Thus, the primary trait required for the application of probiotics in weanling pigs is the ability to inhibit pathogen colonization.

Both 'allochthonous' and 'autochthonous' *Lactobacillus*, which differ in whether share co-evolutionary history with the respective host, have been applied as probiotics in previous studies. [131,148,266,267]. Divergent effects were observed between host-adapted (autochthonous) and non-host-adapted (allochthonous) probiotic *Lactobacillus* in pigs. Inclusion of host-adapted probiotic strain *L. reuteri* TMW 1.656 skewed the assembly of *Lactobacillus* community in swine colon from *L. delbrueckii* to *L. reuteri / L. delbrueckii* dominant [267]. The same strain also effectively reduced pathogen load in weanling pigs [268]. Its ability to produce reutericyclin also favored the control of post-weaning diarrhea by inhibiting the growth of Gram-positive competitor

and modulating the development of fecal microbiota of weanling pigs [23]. And therefore, the determinant contributor to probiotic effects of reutericyclin-producing *L. reuteri* is still uncertain. In contrast, a large number of commercial probiotic strains were allochthonous to pigs and more likely to stimulate immune response against pathogen infection [113,124,125,128–130]. But probiotics tended to induce systematic immunity rather than mucosal specific IgAs in ETEC / Salmonella pigs [124,128,129].

To preserve these commercial probiotic's long-term viability and functionality, freeze drying is a commonly used stabilization strategies in the microbiological industry [269,270]. In animal production, feed fermentation with lactic acid bacteria is also widely used as an economical alternatives, which is expected to give extra benefits to animal due to the released nutrients and endogenous enzymes during fermentation [146,162,163]. However, the effects and differences of these two supplementation methods for the same probiotic strain have not been verified in practical application.

Thus, the aim of this study was to determine the effects of feeding different lactobacilli on pathogen load and gut microbiota dysbiosis in weanling pigs. The experiment was designed to (1) determine the contribution of reutericyclin formation in probiotic function of *L. reuteri*; (2) compare the beneficial effects of lactobacilli with different ecological origins in weanling pigs; (3) evaluate the effects of freeze drying process on probiotic effects of *L. fermetum* and *L. casei.*

5.2. Materials and methods

5.2.1. Microorganisms and growth conditions

Reutericyclin-producing *L. reuteri* TMW1.656 and isogenic reutericyclin-negative *L. reuteri* TMW1.656Δ*rtcN* [225] were routinely grown on modified MRS agar [228] at 37 °C anaerobically. Two commercial probiotics *L. casei* K9-1 and *L. fermentum* K9-2 isolated from dog feces were

provided by CanBiocin Inc. (Edmonton, AB, Canada) and grown under same conditions. Freeze-dried cultures of *L. casei* K9-1 and *L. fermentum* K9-2 with 10^9 CFU / g vegetative cell were also provided by CanBiocin Inc. (Edmonton, AB, Canada), which were stored at 4 °C until use.

5.2.2. Experimental diets and animals

Overnight cultures of *L. reuteri* TMW1.656, *L. reuteri* TMW1.656Δ*rtcN*, *L. casei* K9-1 and *L. fermentum* K9-2 were applied for feed fermentation as previous described [267,271]. The quality of fermentation process was monitored by determining the dominance of inoculated strains and final pH.

Three fermented wheat containing dietary treatments: *L. reuteri* TMW1.656, *L. reuteri* TMW1.656Δ*rtcN* and *L. casei* / *L. fermentum* fermentation, were obtained by mixing 98% basal diet with 2% fermented wheat [272]. Three unfermented wheat containing dietary treatments: control, acidified control and *L. casei* / *L. fermentum* freeze-dried was prepared by mixing 98% basal diet with 2% unfermented wheat, 2% acidified wheat (pH 3.8) and 2% freeze-dried culture powder, respectively. The significant components of these diet treatments were listed in Table 1. The pH values of all the experimental dietary treatments (except control) were stabilized at 5.44 ± 0.09. The average cell counts of *L. reuteri* TMW1.656, *L. reuteri* TMW1.656Δ*rtcN* and *L. casei* / *L. fermentum* (freeze-fried and fermentation) in feed were around 10^7- 10^8 CFU /g [267].

The feeding trial complied all relevant principles regarding animal welfare and was approved by the University of Alberta Animal Care and Use Committee for Livestock. A total of 48 male piglets (Duroc × Large White/ Landrace F1) with similar bodyweight (6-7 Kg) were selected at weaning (21 days of age). Six experimental diets were allocated to 48 piglets with completely randomized block design, which provided 8 replicates per dietary treatment. All pigs were raised as previous described [267,271] and weighted at the end of each week. Diets were provided twice per day with

equal amounts. The content of dry matter (DM) in feed and leftover were measured each day and applied for feed intake calculation. The feed efficiency was calculated from average weight gain (g / day) over average feed intake (g DM / day).

Table 5.1. Significant components of experimental diets used in this study

Components	Control	Acidified control	*L. casei / L. fermentum*		*L. reuteri*	
			Freeze-dried	Fermentation	TMW1.656	TMW1.656*ΔrtcN*
Acid	-	+	+	+	+	+
L. casei / L. fermentum	-	-	+	+	-	-
L. reuteri	-	-	-	-	+	+
Reutericyclin	-	-	-	-	+	-

+ means the presence of component, − means the absence of component

5.2.3. Samples preparation and DNA extraction

Feces were collected from the pen of each animal at days 0, 7, 14 and 21 and stored at -20 °C immediately after sampling. Digesta samples were collected from ileum, caecum, and colon at euthanasia and stored at -20 °C. Total bacterial DNA was extracted from 2-3 g of feces or digesta samples after thawing and mixing the frozen samples. Stool samples were homogenized with pH 7.4 Phosphate Buffered Saline solution (Gibco, ThermoFisher Scientific, Burlington, USA) and pre-treated by bead-beating (BioSpec Products, Inc., Bartlesville, USA) for 30s × 8 times. Treated samples were heated at 95°C for 15 minutes to lyse cell before using QIAamp Fast DNA stool mini kit (Qiagen, Inc., Valencia, CA, USA). Only DNA samples with an A260/280 ratio higher than 1.8 were used for pathogen detection and microbial analysis. Purified DNA was diluted to 50 ng /μL for use.

5.2.4. Intestinal pathogen detection using qPCR and HRM-qPCR

Total *E. coli*, 8 swine ETEC related virulence factor genes, including 5 ETEC fimbriae genes (F6, F18, F41, K88 and K99) and 3 toxin genes (LT, STa and STb), *Clostridium* cluster I and *C. perfringens* α toxin were screened by multiplex HRM-qPCR or / and qPCR in 144 digesta and 191

feces samples. Primers used in this study and their target genes are listed in Table 5.2. Five swine ETEC fimbriae genes were quantified by multiplex HRM-qPCR using Rotor-Gene Q (QIAGEN) HRM-thermo cycler and Type-it HRM Kit (QIAGEN) as previously described [79]. A total volume of 25 µL HRM-qPCR reactions contained 12.5 µL 2× HRM Master Mix, 3 µL template bacterial DNA, 200 nM × 5 targets primers. A standard curve for total content of 5 targets was established from 10-fold dilutions of the mixed standards, containing 2×10^{2} to 2×10^{-8} gene copies / µL of each purified positive control, which were amplified from stool samples with same conditions. The relative abundance of each target was quantified based on the linear correlation between the percentage of melting peak area and relative portion of the respective amplicon in mixed template [79]. Total *E. coli,* ETEC toxin gens LT, Sta and STb, *Clostridium* cluster I and *C. perfringens* were determined by qPCR using 7500 Fast real-time PCR system (Thermo Fisher Scientific, Burlington, USA). qPCR conditions were applied as previous described [23,79,271].

Table 5.2. Primers used in this study

Target gene	Sequence (5'→ 3') (name)	Size (bp)	Tm (°C)*
K88 fimbriae (*fae G*)	GCACATGCCTGGATGACTGGTG (K88 F) CGTCCGCAGAAGTAACCCCACCT (K88 R)	439	63
K99 fimbriae (*fan A*)	CACTTGAGGGTATATGCGATCTT (K99 F) GACCTCAGTCACAGCAACTATAC (K99 R)	92	62
F6 fimbriae (*Fas A*)	GTTCCAGCCTCCAATGATACT (F6 F) GAAAGAGCTAATCCGCCATTTG (F6 R)	128	62
F41 fimbriae Sub-unit A	GACCTCAGTCACAGCAACTATAC (F41 F) CGACCCGCAACATCCTTATT (F41 R)	110	62
F18 fimbriae (*Fed A*)	GGAGGTTAAGGCGTCGAATAG (F18 F) CCACCTTTCAGTTGAGCAGTA (F18 R)	90	62
Universal stress protein A	CCGATACGCTGCCAATCAGT (UspA F) ACGCAGACCGTAGGCCAGAT (UspA R)	884	66
Clostridium cluster I	GTGAAATGCGTAGAGATTAGGAA (CI F) GATYYGCGATTACTAGYAACTC (CI R)	665	58
C. perfringens α-toxin	CTTGGAGAGGCTATGCACTATTT (CPα F) CTTAACATGTCCTGCGCTATCA (CPα R)	90	60
Heat-labile enterotoxin	CCGTGCTGACTCTAGACCCCCA (LT F) CCTGCTAATCTGTAACCATCCTCTGC (LT R)	480	68
Heat-stable enterotoxins a	ATGAAAAAGCTAATGTTGGC (STa F) TACAACAAAGTTCACAGCAG (STa R)	193	65
Heat-stable enterotoxins b	TGCCTATGCATCTACACAAT (STb F) CTCCAGCAGTACCATCTCTA (STb R)	113	60

5.2.5. Fecal microbiological analysis using 16S rRNA gene sequencing

A total of 191 genomic DNA isolates from feces was sequenced on pair end Illumina MiSeq platform (2 × 300 bp) by amplifying the V5-V6 domain of the 16S rRNA gene (University of Minnesota Genomics Center, Minneapolis, MN, USA). The forward and reverse primers sequences used for amplification were listed as following: GTGCCAGCMGCCGCGGTAA and CGACRRCCATGCANCACCT.

A total of 6,647,893 16S rRNA gene sequences, or an average 34,805 sequences per sample with an average length of 266 bp passed quality control from QIIME pipeline (MacQIIME 1.9.1-20150604) [233]. UCLUST was applied for OTUs (Operational Taxonomic Units) clustering with at least 97% similarity [273]. Only OTUs with relative abundance > 0.005% were retained for downstream analysis. After chimera checking and filtering, taxonomy was assigned to OTUs at genus or lower level with 97% or less average nucleotide identity (ANI) by aligning to GreenGenes reference database. Taxonomy information was further categorized into different levels by *summarize_taxa_through_plots.py*. Principle coordinates analysis (PCoA) and analysis of similarities (ANOSIM) were performed to explore microbial community differences through weighted UniFrac distance matrix.

5.2.6. Statistical analysis

The data for growth performance (average feed intake, average daily gain and feed efficiency), intestinal pathogen load (Log (gene copy no./g) of *Clostridia* Cluster I, *C. perfringens* α toxin, *E. coli* and ETEC virulence factors) were analyzed by linear mixed-effects model fitted for randomized block design in R (version 3. 5.1, The R Foundation for Statistical Computing, 2018). In this model, pigs were as considered as experimental units; Time, dietary treatments, as well as time and dietary treatments were calculated as fixed factors; The difference between blocks were

regarded as random effects. The normality and homogeneity of all variables were determined by Shapiro-Wilk normality test and Bartlett test, respectively. The date for relative abundance of OTUs and alpha diversity were analyzed by Kruskal-Wallis rank-sum test in MacQIIME 1.9.1 [233]. The pairwise comparisons were performed with Wilcoxon rank sum test. Analysis of similarities (ANOSIM) for ETEC virulence factors data was calculated by Bray–Curtis dissimilarity; Principle component analysis (PCA) for ETEC virulence factors data was analyzed using "kassambara/factoextra" packages in R. ANOSIM and Principle coordinate analysis (PCoA) for 16S rRNA sequence data was calculated from weighted UniFrac distance matrix. Results are presented as means ± standard deviation. P values < 0.05 with Bonferroni-adjustment were considered significant.

5.3. Results

5.3.1. Animal health and growth performance of pigs

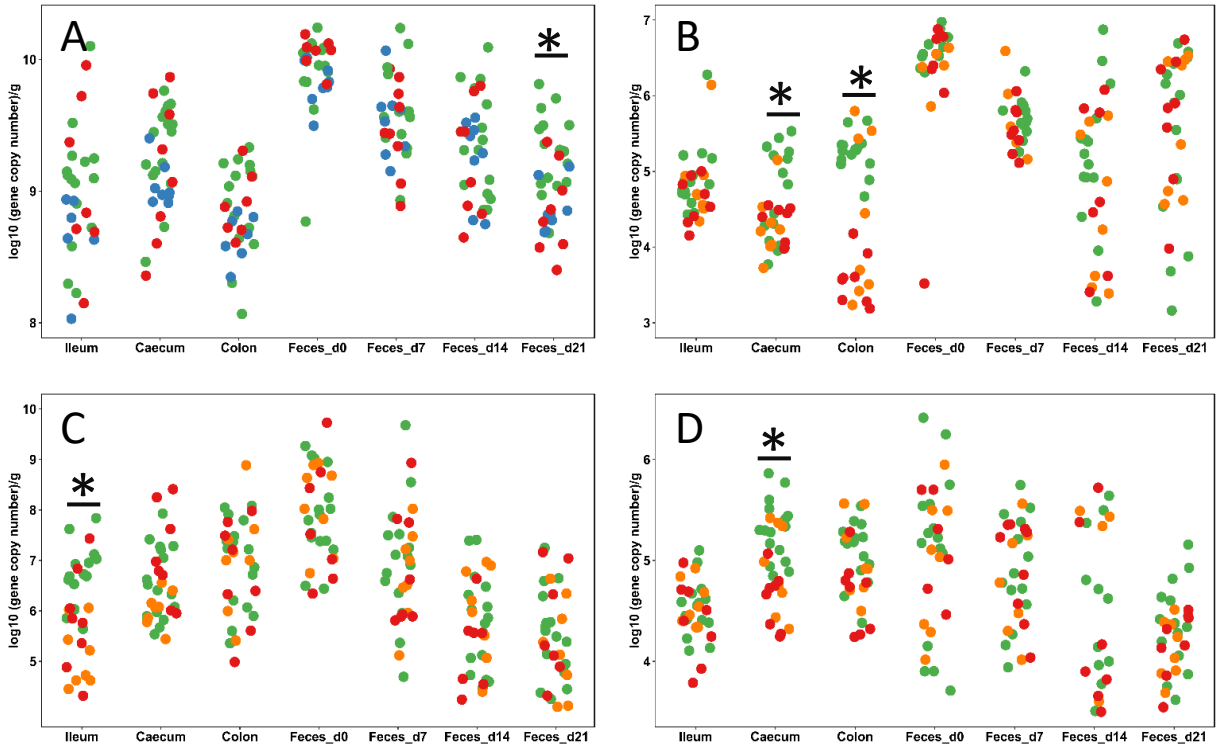
All pigs remained healthy and no pigs exhibited persistent diarrhea during the 3 weeks trial. The parameters reflecting growth performance are listed in Table S5.1. The average feed intake and daily gain increased with time corresponding to feed efficiency increased from 0.65kg to 0.8kg in the first two weeks and remained stable in the third week (data no show). No significant difference in feed efficiency was detected between dietary treatments.

5.3.2. Reduction of pathogens and toxins in weanling pigs by feeding probiotic lactobacilli

qPCR and HRM-qPCR were applied to determine the gene content of *Clostridia* Cluster I, *C. perfringens* α toxin and ETEC virulence factors, including LT, heat stable toxin a (STa), heat stable toxin b (STb), heat labile toxin (LT), K99, F18, F41, K88 and F6 fimbriae in weanling pigs. Feces were weekly sampled and ileum, caecum and colon digesta were collected at day 21. The overall concentration of these pathogens and virulence factors were lower in ileum than that in caecum and colon (Figure 5.1 & Table S5.2) and decreased over time in feces. The gene content of

Clostridia Cluster I was detectable in swine digesta samples but did not differ across dietary treatments (Figure 5.1 & Table S5.2). While feeding freeze-dried *L. casei / L. fermentum* or *L. reuteri* TMW1.656 *ΔrtcN* significantly ($P < 0.05$) reduced *Clostridium* Cluster I in feces at day 21. Differently, feeding *L. reuteri* TMW1.656 or *L. reuteri* TMW1.656 *ΔrtcN* decreased *C. perfringens* α toxin in caecum and colon (Figure 5.1B & Table S5.2).

Figure 5.1. Reduction of *Clostridium* cluster I (A), *C. perfringens* α toxin (B), total *E. coli* (C) and heat-labile toxin (D) by feeding lactobacilli. Digesta samples were collected from ileum, caecum and colon at day 21. Feces were collected at day 0, 7, 14 and 21. All the specimens were analysed by qPCR with specific primers. Each dot represents individual fecal samples, colored according to the dietary treatments: Control and Acidified control (green, n = 16), freeze-dried *L. casei / L. fermentum* (blue, n=8), fermented *L. casei / L. fermentum* (purple, n =8), *L. reuteri* TMW1.656 (orange, n=8) and *L. reuteri* TMW1.656 *ΔrtcN* (red, n =8). Data with asterisk (*) are significantly different (P <0.05) between dietary groups. *L. casei / L. fermentum* diet groups are not shown in panels B, C, and D because the gene copy numbers were not different from the control.



Total *E. coli* in ileum, LT in caecum and *C. perfringens* α toxin in caecum and colon decreased in pigs fed with *L. reuteri*. Isogenic reutericyclin-negative *L. reuteri* also lowered ($P < 0.05$) *C. perfringens* α toxin and LT in caecum and colon compared to control or acidified control diet. The addition of *L. casei* / *L. fermentum* (freeze-fried and fresh fermentation) did not significantly alter the pathogen load in digesta and feces except for reducing *C.* Cluster I in feces by freeze-dried *L. fermentum* and *L. casei* at day 21. The content of other ETEC virulence factors did not differ among diet groups but varied with aging.

5.3.3 Longitudinal changes of ETEC virulence factors in swine feces during the first 21 days after weaning

A longitudinal profile of ETEC virulence factors through the 3 weeks trial was exhibited during the first 21 days after weaning (Figure 5.2). The load of these pathogens was low at weaning and then displayed a chaotic profile during the first week followed by a steadily decreasing during the following two weeks (Figure 5.2). LT significantly decreased after the first week while STa were transitionally higher at day 7 ($P < 0.05$). F18 and F6 fimbriae significantly increased the predominant ETEC fimbriae type detected in weanling pigs after day 7. Other fimbriae types and STb toxin were detected in feces with low abundance ($< 10^5$ copies / g) (Figure 5.2 & Table S5.3) and no significant difference was observed among different time points. PCA also demonstrated similar variations in abundance and combinations of ETEC virulence factors (Figure 5.3). ANOSIM suggested that sow or litter effects significantly influenced the patterns of ETEC virulence factors during the first week while time significantly contributed to the variations through the experimental period (Table 5.3).

Table 5.3. ANOSIM a of Bray–Curtis dissimilarity calculated with ETEC virulence factor genes content in feces at 0, 7, 14 and 21 days after weaning

Time / Factor	Time		Diet		Sow		Block	
	R[b]	*P* value	R	*P* value	R	*P* value	R	*P* value
Day 0	*NA*	*NA*	*NA*	*NA*	0.068	0.128	-0.046	0.954
Day 7	*NA*	*NA*	-0.033	0.909	***0.403***	***0.001***	-0.018	0.636
Day 14	*NA*	*NA*	-0.031	0.902	0.036	0.272	0.015	0.318
Day 21	*NA*	*NA*	0.035	0.111	0.0.42	0.247	-0.011	0.604
Overall	***0.22***	***0.001***	-0.006	0.817	-0.002	0.531	-0.001	0.537

[a]ANOSIM, Analysis of similarity

[b]An R value near +1 means that there is dissimilarity between the groups, while an R value near 0 indicates no significant dissimilarity between the groups

Figure 5.2. Dynamic profiles of ETEC virulence factors at 0, 7, 14 and 21 days after weaning. All the samples were analyzed by qPCR with specific primers. Each dot represents individual fecal samples, colored according to the dietary treatments: Control and Acidified control (green, n = 16), freeze-dried *L. casei / L. fermentum* (blue, n=8), fermented *L. casei / L. fermentum* (purple, n =8), *L. reuteri* TMW1.656 (orange, n=8) and *L. reuteri* TMW1.656 *ΔrtcN* (red, n =8). Data with asterisk (*) are significantly different (P <0.05) between dietary groups.

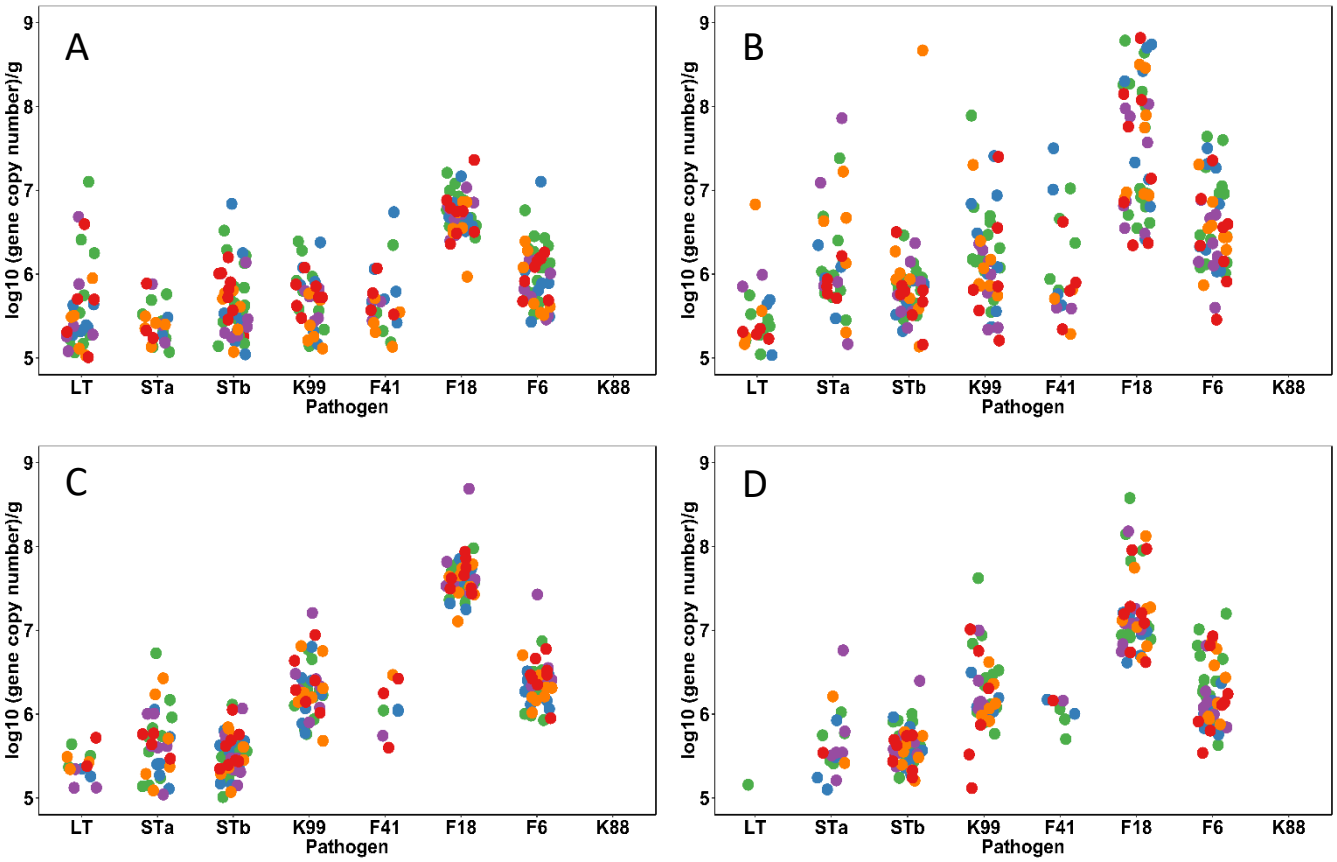
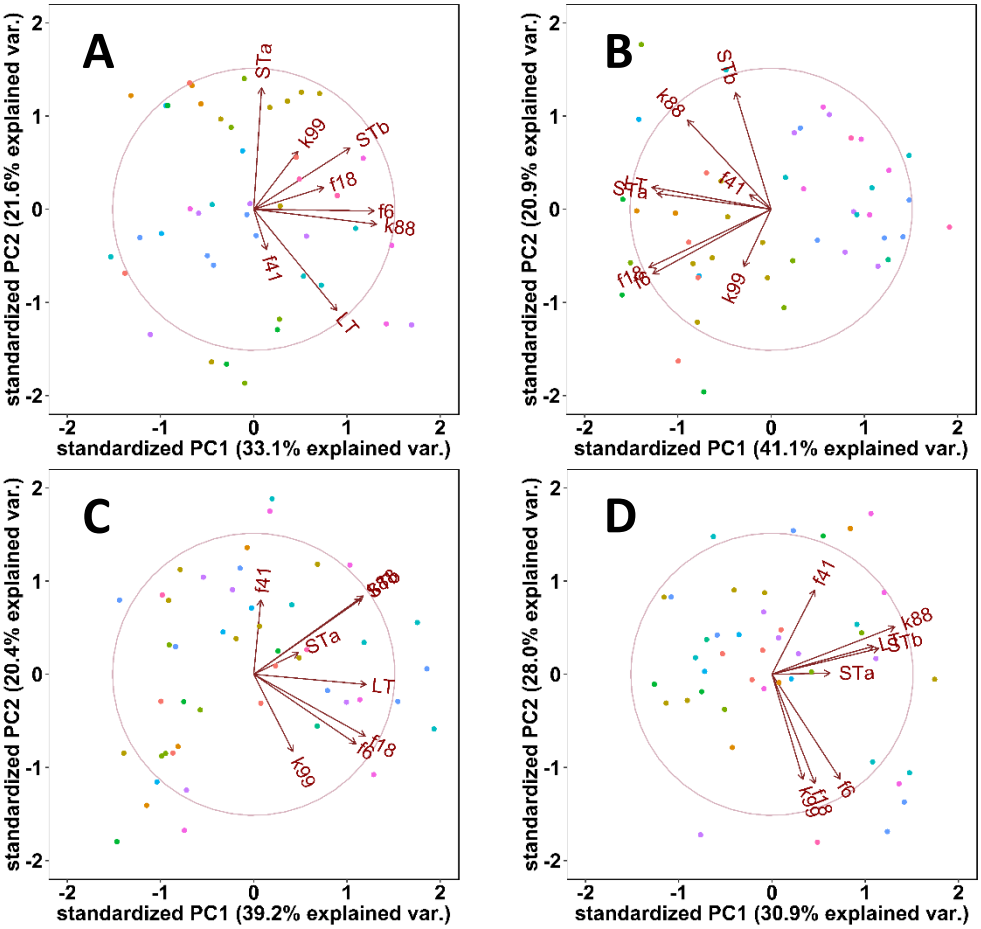


Figure 5.3. Principle component analysis of ETEC virulence factors at 0, 7, 14 and 21 days after weaning. Each dot represents individual fecal samples, colored by sow: dots with same color are born from the same sow. The arrows represent the original variables, where the corresponding directions represent the correlations between the original variables and the principal components, and the length represents the contribution of the original data to the principal component.



5.3.4. Alterations in fecal microbiota and *Lactobacillus* community by feeding lactobacilli in weanling pigs

To determine the potential action mode of probiotic, effects of probiotic lactobacilli on α-diversity (Figure 5.4) and β-diversity (Table 5.4 & Figure S5.1) of fecal microbiota and *Lactobacillus* were assessed in weanling pigs. Observed bacterial types and α-diversity of fecal microbiota

significantly increased in the first two weeks and plateaued in the last week (Figure 5.4). Pigs fed with acidified wheat were noted for the reduction of bacterial types and α-diversity ($P < 0.05$). But the observed types and α-diversity of *Lactobacillus* illustrated an inverse trend that was significantly decreased in the first week ($P < 0.05$) and remained stable during the following two weeks. For the dietary effects, the types of *Lactobacillus* transiently reduced within the first week ($P < 0.05$) only in pigs fed with acidified wheat.

The dissimilarities between pigs fed with different lactobacilli were further compared by the analysis of β-diversity (Table 5.4 & Figure S5.1). The overall fecal microbiota of weanling pigs was differentiated by feed phase and litters ($P < 0.05$, Table 5.4). *Lactobacillus* community was also significantly altered ($P < 0.05$) by increasing wheat content but not influenced by litter effects ($P = 0.166$). The inclusion of probiotic lactobacilli in feed did not change the structure of fecal microbiota ($P = 0.683$) but significantly contributed to the difference of *Lactobacillus* community ($P < 0.05$). Moreover, dietary effect was observed in shaping *Lactobacillus* composition ($P < 0.05$).

Figure 5.4. Fecal microbiota and *Lactobacillus* diversity during the first 3 weeks after weaning. Total number of bacteria (A) or *Lactobacillus* (B) types observed and Shannon diversity index of fecal microbiota (C) or *Lactobacillus* community(D) were analyzed based on partial 16S rRNA sequences. Each box represents one dietary treatment colored as following: Control (light red, n =8), Acidified control (brown, n = 16), freeze-dried *L. casei / L. fermentum* (green, n=8), fermented *L. casei / L. fermentum* (cyan, n =8), *L. reuteri* TMW1.656 (light blue, n=8) and *L. reuteri* TMW1.656 *ΔrtcN* (plum, n =8). Indexes with different letters are significantly different (P <0.05) between time points.

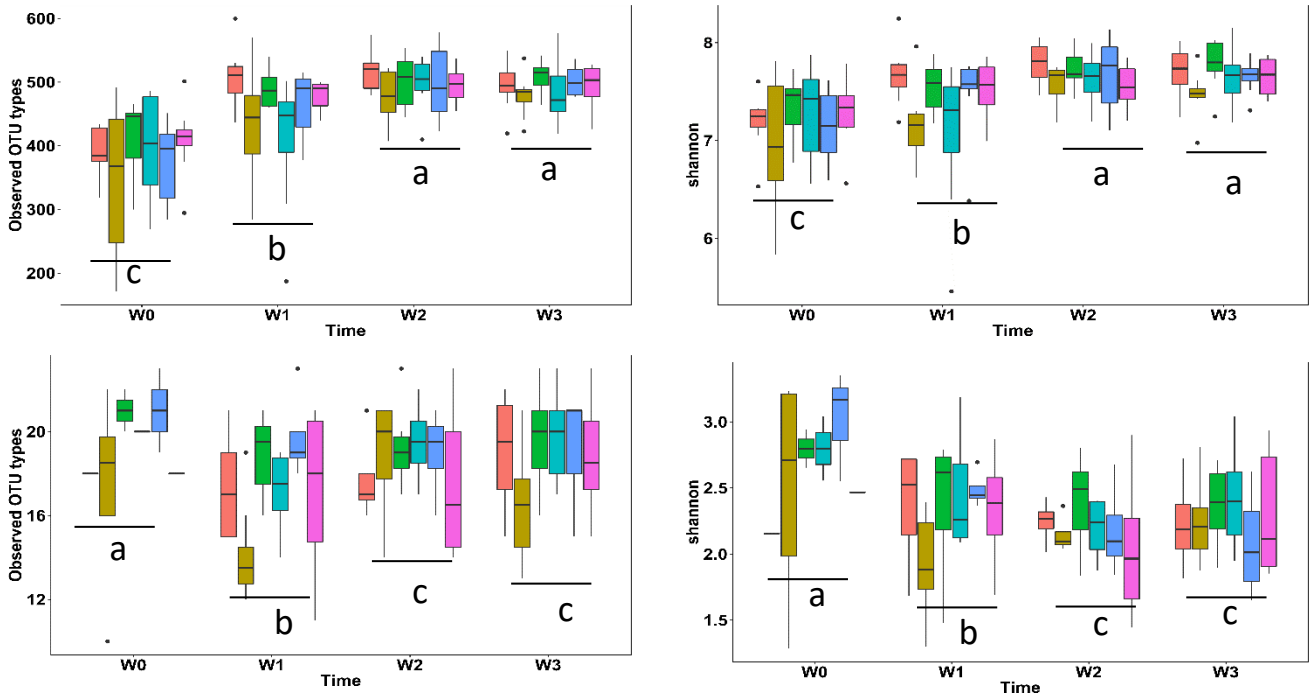


Table 5.4. ANOSIM of weighted UniFrac distance matrix calculated with partial 16S rRNA sequences

Factor	Df	Fecal microbial community		*Lactobacillus* community	
		R value	*P* value	R value	*P* value
Acid	1	-0.062	0.847	-0.013	0.609
Diet	5	0.008	0.125	0.025	***0.028***
Feed phase	2	0.505	***0.001***	0.135	***0.001***
Litter	10	0.100	***0.001***	0.016	0.166
Strain	2	-0.003	0.683	0.035	***0.008***

5.3.5. Contribution of feeding lactobacilli in restoring fecal microbiota from weaning dysbiosis

Based on structure analysis of fecal microbiota, relative abundance of major bacteria was further compared to determine the effects of lactobacilli on microbial restoration after weaning (Table 5.5 & 5.6). Increased abundance was mainly detected in genus *Bifidobacterium* and abundant genera from phyla *Bacteroidetes, Firmicutes, Tenericutes* and *WPS-2* while most genera from phylum *Proteobacteria* decreased over time. Compared to acidified control diet, feeding *L. fermentum* and *L. casei* increased the abundance of *Bifidobacterium* and *Veillonellaceae at day 7* and decreased *Succinivibrio* and *Pirellulaceae* at day 14 ($P < 0.05$). Influence of *L. reuteri* on fecal microbiota was only observed in decreasing *Enterobacteriaceae* ($P < 0.05$).

5.4. Discussion

Immunomodulation and competitive colonization are the two major mechanism of probiotics to inhibit pathogen infection. The ecological origin of the probiotic strain was suggested to determine whether one or the other is more important [9,13,14,16,18]. This study observed reduction of *E. coli* and ETEC toxins mainly in pigs supplied with host-adapted *L. reuteri*. Anti-ETEC effect was also reported in studies applying host-adapted *L. amylovorus* (*L. sobrius*) and higher dose of *L.*

reuteri [23,197,274,275]. In contrast, the nomadic organisms *L. fermentum* and *L. casei* decreased the abundance of *Clostridium* cluster I but not of *E. coli*. A possible explanation for this divergent effect is that host-adapted *L. reuteri* eliminated ETEC colonization through exclusively competition while nomadic *L. fermentum* and *L. casei* inhibited *Clostridum* spp. by stimulating systemic immunity; however, differential effects of host adapted and nomadic lactobacilli on intestinal microbial ecology and host immunity remain to be validated in future studies. Reasonable viability and stable immunomodulation of probiotics under appropriate drying process facilitated the development of pharmabiotic [269,276,277]. While equivalent probiotic protections provided by feed / food fermentation promoted their advantages in daily life and animal production with lower cost and liberated from processing technology [23,161,278].

The inclusion of probiotic lactobacilli exerted limited effects on fecal microbiota of weanling pigs. The initial composition of fecal microbial community varied between litters and was then reshaped by feed over time, which agreed with significance of diet in modulating development of swine gut microbiota [279]. Reutericyclin-producing *L. reuteri* reduced *Enterobacteriaceae* in this study and transiently inhibited lactobacilli [267]. Bacteriocin expression in commensal bacteria or lactobacilli was suggested favoring their competition for ecological niche in host mammals or sourdough fermentation [280,281]. However, lower density bacteriocin sequence in human colonic microbiome implied less relevancy of bacteriocin production in the stability of microbiota in colon than in other body sites, such as in the oral cavity and the vagina [280,282,283]. Subtle impact of bacteriocin production in *L. salivarius* was also observed on mouse and pig gut microbiota. Although stronger restraint was elicited in previous study with higher dose of the same *L. reuteri* strain [23], we concluded no inhibitory effects of reutericyclin on Gram-positive bacteria referred from the limited difference detected between *L. reuteri* and its reutericyclin-negative

isogenic mutant in this study. We also suggested the need for isogenic control to validate the influence of bacteriocin expression in probiotics on gut microbiota [284].

In the primary cause of PWD, decades of research on ETEC infection didn't identified specific strain responsible for triggering diarrheal onset. Different from the strain specificity analysis, our high-resolution ETEC virulence factor spectrums well described the variable pathogenicity of ETEC in weanling pigs, which provided a clearer basis for the prevention of swine ETEC infection by vaccines or fimbriae accepter analogues. We agreed the higher relevancy of F18 fimbriae in weanling pigs and decreasing abundance of LT and increase of Sta after weaning. Few ETEC strains additionally carry Stx2e, which are referred as hybrid Shiga toxin-producing *E. coli* /ETEC (STEC/ETEC) but was not included in our detection spectrum [178]. The absence of F18 receptor in neonatal pigs and high frequency combination of Stb /F18 and LT /K88 contributed to the variable virotypes of swine ETEC. Piglets from the same sow harbored more similar profile of virulence factors during the first week after weaning, suggesting that early colonization of ETEC was relevant to maternal-neonatal transmission and persisted shortly after weaning [30]. ETEC become more virulent after passage through intestinal tract. Strains that colonize from the sow intestinal tract are therefore more virulent than strains from an environmental source [285]. Time or age significantly contributed to the variations in patterns of ETEC virulence factor and microbiota assembled by 16 rRNA gene sequencing in weanling pigs [279]. Therefore, it was never accidental that the longitudinal profiles of ETEC virulence factor coincided with development of gut microbiota in weanling pigs. In previous studies, the abundance of *Bacteroidetes*, including genera from *Prevotellaceae*, *Lachnospiraceae* and *Ruminococcaceae* families were higher in health pig than that in diarrheal pigs [51,286], which positively correlated with the increasing colonization of *Bacteroidetes* after weaning [287] . Thus, promoting an earlier

colonization of *Bacteroidetes* could be regarded as a strategy to decease the incidence of ETEC infection in early-weaned pigs.

In conclusion, early colonization of ETEC was associated with, but not limited to maternal-neonatal transmission, which necessitate the significance of maintaining sanitary environment to decrease ETEC load and virulence in sow and suckling pigs. Host-adapted probiotic lactobacilli exerted stronger ETEC elimination, which provided alternative protections for early weaned piglets. Strategies promoting an earlier colonization of *Bacteroidetes* also was positively relevant to ETEC reduction in pigs after weaning.

Table 5.5. Relative abundance (%) of bacterial genera in fecal microbiota of pigs during the first 3 weeks after weaning, determined by sequencing of 16S rRNA tags. Data were analyzed by QIIME pipeline and are represented as mean ± SD. Data in the same row that do not share a common superscript are significantly different ($P < 0.05$).

Genus	Day 0	Day 7	Day 14	Day 21
ACTINOBACTERIA				
Bifidobacterium	ND	ND	0.01±0.03 ^{b}	0.12±0.79 ^{a}
[F:Coriobacteriaceae]	0.94±1.12 ^{b}	1.20±1.13 ^{a}	0.73±0.33 ^{b}	1.16±0.51 ^{a}
BACTEROIDETES				
Prevotella	6.36±4.56 ^{c}	7.12±2.96 ^{c}	10.15±3.96 ^{b}	12.89±4.96 ^{a}
[F:S24-7]	5.41±4.37 ^{b}	6.60±3.94 ^{ab}	6.08±2.97 ^{b}	7.65±3.86 ^{a}
CF231	0.11±0.10 ^{c}	0.31±0.21 ^{b}	0.84±0.58 ^{a}	0.81±0.44 ^{a}
Other *[O:Bacteroidales]*	11.81±6.22 ^{a}	5.39±3.03 ^{c}	7.00±2.64 ^{b}	6.14±2.56 ^{b}
FIRMICUTES				
Lactobacillus	3.87±4.20 ^{b}	11.31±9.25 ^{a}	8.43±4.76 ^{a}	8.70±4.12 ^{a}
Enterococcus	0.04±0.22 ^{b}	0.87±2.97 ^{a}	0.02±0.08 ^{b}	0.06±0.19 ^{b}
Streptococcus	0.23±0.42 ^{a}	0.05±0.13 ^{b}	0.02±0.10 ^{c}	0.04±0.11 ^{bc}
Blautia	0.37±0.42 ^{c}	1.59±2.17 ^{b}	1.95±1.45 ^{a}	1.61±0.73 ^{a}
Coprococcus	0.06±0.05 ^{c}	1.09±0.84 ^{a}	1.33±0.99 ^{a}	0.77±0.67 ^{b}
[F:Ruminococcaceae]	16.63±3.82 ^{a}	14.59±3.92 ^{b}	16.80±3.34 ^{a}	13.61±2.61 ^{b}
[F:Lachnospiraceae]	9.45±2.99 ^{a}	9.58±3.63 ^{a}	9.36±3.38 ^{a}	7.90±1.99 ^{b}
Turicibacter	0.48±0.34 ^{a}	0.26±0.75 ^{ab}	0.16±0.62 ^{ab}	0.20±0.33 ^{b}
Erysipelotrichaceae	3.51±2.15 ^{a}	2.12±1.37 ^{ab}	1.78±0.71 ^{b}	1.85±0.58 ^{ab}
[F:Clostridiaceae]	1.47±0.64	1.76±1.91	1.38±1.92	1.52±1.13
[F:Veillonellaceae]	0.70±0.93 ^{c}	1.47±1.67 ^{b}	1.70±1.84 ^{b}	3.58±2.63 ^{a}
[F:Mogibacteriaceae]	0.60±0.46 ^{b}	1.10±1.35 ^{a}	0.87±0.49 ^{a}	0.53±0.26 ^{b}
Other *[O:Clostridiales]*	10.81±3.93 ^{a}	8.97±4.16 ^{ab}	8.13±2.41 ^{b}	7.40±2.12 ^{b}
PROTEOBACTERIA				
Oxalobacter	0.10±0.07 ^{a}	0.04±0.03 ^{c}	0.11±0.05 ^{a}	0.12±0.06 ^{a}
Desulfovibrio	0.46±0.39 ^{a}	0.06±0.07 ^{c}	0.12±0.09 ^{b}	0.11±0.09 ^{bc}
[F:Enterobacteriaceae]	1.23±2.24 ^{a}	0.93±2.21 ^{ab}	0.12±0.33 ^{c}	0.40±1.02 ^{bc}
Succinivibrio	0.33±1.13 ^{b}	0.73±1.30 ^{a}	0.11±0.20 ^{b}	0.20±0.32 ^{b}
Other	0.58±1.27 ^{a}	0.27±0.75 ^{b}	0.30±0.34 ^{a}	0.26±0.30 ^{b}
PLANCTOMYCETES				
[F:Pirellulaceae]	1.35±1.42 ^{b}	1.95±2.27 ^{ab}	2.14±2.53 ^{a}	1.65±1.25 ^{ab}
SPIROCHAETES				
Treponema	1.85±1.53 ^{ab}	2.33±1.88 ^{ab}	2.55±2.59 ^{a}	1.59±1.51 ^{b}
TENERICUTES				

[O:RF39]	6.21±4.09	7.11±3.32	6.16±2.60	6.69±2.69
WPS-2	0.46±1.56 [b]	0.41±0.99 [b]	1.07±1.69 [a]	1.35±1.46 [a]
OTHER	3.87±4.08 [a]	2.35±2.74 [b]	1.65±2.40 [bc]	1.01±1.54 [c]
Unassigned	4.75±2.89 [c]	5.53±2.75 [b]	6.46±2.54 [ab]	7.87±3.53 [a]
Total	94.03±2.46	97.08±1.66	97.55±1.48	97.78±1.06

ND means not detected. Unassigned genera are presented with upper level of family (F) or order (O) in square brackets. “Unassigned” means a good hit to a poorly defined taxonomy sequence. “Other” means the assignment is ambiguous

Table 5.6. Bacterial genera with significant difference ($P < 0.05$) in relative abundance (%) of fecal microbiota of the pigs feed with experimental diets during the first 3 weeks after weaning, determined by sequencing of 16S rRNA tags. Data were analyzed by QIIME pipeline and are represented as mean ± SD.

Genus	Acidified Control	Freeze dried *L. fermentum* & *L. casei*	*L. fermentum* & *L. casei*	*P* value	*L.reuteri* TMW 1.656	*L.reuteri* TMW 1.656 Δ*rtcN*	*P* value
Day 7							
Bifidobacterium	ND	ND	0.02±0.04	**0.04**	ND	ND	1.00
Enterococcus	0.49±0.85	0.43±0.82	1.15±2.88	0.44	2.40±6.72	0.40±0.73	0.05
[F:Veillonellaceae]	0.45±0.36	2.20±1.81	0.96±0.93	**0.04**	2.39±1.98	1.62±2.56	0.06
[F:Enterobacteriaceae]	0.10±0.20	0.33±0.69	0.09±0.09	0.83	0.07±0.08	ND	**0.01**
Day 14							
Succinivibrio	0.28±0.36	0.04±0.12	0.06±0.09	**0.04**	0.07±0.08	0.18±0.24	0.29
[F:Pirellulaceae]	1.88±3.19	1.44±1.12	3.34±2.36	**0.04**	2.66±4.25	0.91±0.92	0.20
Day 21							
[o__RF39]	5.65±1.81	9.07±2.89	6.16±2.45	0.05	7.44±3.09	6.29±2.52	0.44

Table S5.1. Growth performance of pigs during the first 3 weeks after weaning

Dietary treatment	Average feed intake (g DM [a] /day)	Average daily gain (g / day)	Feed efficiency (G / F)
Control	499±20.4	406±72.3	0.81±0.13
Acidified control	485±25.3	343±78.1	0.70±0.14
L. casei / L. fermentum			
Freeze-dried	493±15.9	378±49.0	0.77±0.10
Fermented	514±83.9	376±86.9	0.74±0.19
L. reuteri			
TMW1.656	489±29.1	383.91±75.98	0.78±0.14
TMW1.656*ΔrtcN*	489±16.8	381±49.6	0.78±0.10
P value [b]			
Acid	0.61	0.11	0.08
L. casei / L. fermentum	0.82	0.90	0.63
L. reuteri	0.05	0.74	0.14
Fermentation	0.09	0.95	0.28
Reutericyclin	0.51	0.93	0.80

[a] DM, dry matter.

[b] For acid effects, *P* values are for the control diet versus the chemically acidified diet; For fermentation, *P* values are for the freeze-dried *L. casei / L. fermentum*-containing diet versus fermented *L. casei / L. fermentum*-containing diet; For *L. casei / L. fermentum*, *P* values are for the comparisons between *L. casei / L. fermentum*-containing diets (freeze-dried and Fermented) and unfermented diets (Control and Acidified control); For *L. reuteri*, *P* values are for the *L. reuteri*-containing diets (TMW1.656 and TMW1.656*ΔrtcN*)versus unfermented diets (Control and Acidified control); For reuterancyclin, *P* values are for the *L. reuteri*-containing diets (TMW1.656 and TMW1.656*ΔrtcN*)

Table S5.2. Gene copy number for *Clostridium* Cluster I, *C. perfringens* α toxin I, LT, STa, STb, *E. coli*, K99, F41, F18, F6 and K88 fimbriae in digesta collected from ileum, caecum and colon at day 21 [a]

Bacterium or bacterial toxin and segments	Log (gene copy no./g) for the following diet:					
	Control	Acidified control	*L. casei / L. fermentum* freeze-dried	*L. casei / L. fermentum*	*L. reuteri* TMW1.656	*L. reuteri* TMW 1.656 Δ*rtcN*
Clostridium Cluster I						
Ileum	9.01±0.23 ^{B}	8.81±0.97 $^{A, B}$	8.42±0.53 ^{B}	8.66±0.69 ^{B}	8.73±0.77	8.96±0.76
Caecum	9.38±0.23 ^{A}	9.26±0.46 ^{A}	9.04±0.17 ^{A}	9.16±0.54 ^{A}	9.04±0.40	9.17±0.55
Colon	8.97±0.40 ^{B}	8.68±0.49 ^{B}	8.57±0.28 ^{B}	8.75±0.41 $^{A, B}$	8.73±0.42	8.70±0.55
C. perfringens α toxin						
Ileum	4.71±0.18 ^{B}	5.08±0.56	4.79±0.25^{B}	4.79±0.36 ^{B}	4.85±0.56	4.65±0.31^{A}
Caecum	4.21±0.33 $^{Y, A}$	5.58±0.89 ^{X}	5.33±0.19 $^{X, A}$	5.38±0.32 $^{X, A}$	4.28±0.43 ^{Y}	4.30±0.25 $^{Y, A}$
Colon	5.34±0.15 $^{X, A}$	5.39±0.76 ^{X}	5.00±0.51 $^{X, Y, A, B}$	4.63±0.32 $^{XY, B}$	4.39±1.06 ^{Y}	< 4 $^{Z, B}$
LT						
Ileum	4.44±0.20 ^{C}	4.61±0.32 ^{C}	4.37±0.34 ^{B}	4.54±0.33 ^{C}	4.57±0.23 ^{B}	4.43±0.43
Caecum	5.07±0.18 $^{XYZ, B}$	5.53±0.20 $^{X, A}$	5.44±0.24 $^{XY, A}$	5.61±0.17 $^{X, A}$	4.91±0.43 $^{Y, A, B}$	4.61±0.29 ^{Z}
Colon	5.26±0.15 $^{X, A}$	4.93±0.32 $^{X, Y, B}$	5.10±0.43 $^{XY, A}$	5.13±0.28 $^{XY, B}$	5.02±0.40 $^{XY, A}$	4.62±0.36 ^{Y}
STa						
Ileum	< 4^{C}	< 4 ^{B}	< 4 ^{B}	< 4 ^{B}	< 4 ^{B}	< 4 ^{B}
Caecum	5.64±0.46 ^{A}	< 4 ^{A}	4.78±2.13 ^{A}	5.66±1.77 ^{A}	5.88±0.82 ^{B}	5.71±0.26 ^{A}
Colon	4.88±0.41 ^{B}	< 4 ^{B}	< 4 ^{B}	4.24±1.82 ^{A}	4.15±1.46 ^{B}	< 4^{B}
STb						
Ileum	4.04±0.41 ^{B}	4.12±0.20 ^{B}	< 4 ^{B}	< 4 ^{B}	4.25±0.40	4.05±0.43
Caecum	< 4 $^{Y, B}$	4.70±0.21 $^{X, A}$	4.80±0.82 $^{X, A}$	4.67±0.12 $^{X, A}$	< 4 ^{Y}	4.02±0.29 ^{XY}
Colon	4.51±0.28 ^{A}	4.13±0.50^{B}	4.63±0.80 ^{B}	4.45±0.38 ^{A}	4.24±0.66	< 4
E. coli						
Ileum	6.91±0.43 ^{X}	6.49±0.73 $^{X, Y}$	6.00±1.23 $^{X, Y}$	6.21±0.97 $^{X, Y}$	5.14±0.64 $^{Y, C}$	5.78±1.08 $^{XY, B}$
Caecum	6.77±0.56	6.36±0.84	5.63±1.51	6.02±0.85	6.04±0.35 $^{Y, B}$	7.09±0.93 ^{A}
Colon	7.33±0.67	6.58±0.95	5.66±1.23	5.74±0.69	7.06±1.05 ^{A}	6.65±1.02 ^{A}
K99 fimbriae						
Ileum	< 4 ^{B}	4.00±0.25 ^{B}	< 4	4.05±0.15 ^{B}	< 4 ^{B}	< 4 ^{B}
Caecum	4.91±0.19^{A}	4.89±0.24^{A}	4.32±0.57	4.61±0.20^{A}	4.67±0.31^{A}	4.56±0.27^{A}
Colon	< 4 ^{B}	< 4 ^{B}	4.01±0.44	4.07±0.12 ^{B}	< 4 ^{B}	< 4 ^{B}
F18 fimbriae						
Ileum	< 4 ^{B}	4.15±0.27 ^{B}	4.16±0.33	4.03±0.25	< 4 ^{B}	4.08±0.21 $^{A, B}$
Caecum	4.86±0.20 ^{A}	4.74±0.28 ^{A}	4.20±0.65	< 4	4.46±0.79 ^{A}	4.33±0.39 ^{A}
Colon	< 4 ^{B}	4.09±0.40 ^{B}	4.11±0.35	4.03±0.19	< 4 ^{B}	< 4 ^{B}
F6 fimbriae						
Ileum	< 4 ^{C}	4.19±0.17 ^{B}	4.06±0.22	< 4 ^{B}	4.09±0.16 ^{B}	4.25±0.17 ^{A}
Caecum	5.10±0.16 ^{A}	5.12±0.24 ^{A}	4.34±0.56 ^{Z}	4.65±0.26 ^{A}	4.90±0.36 ^{A}	4.45±0.50 ^{A}
Colon	4.07±0.30 ^{B}	4.27±0.25^{B}	< 4	4.05±0.12 ^{B}	< 4^{C}	< 4 ^{B}

a Data are presented means ± SD (n=48). Superscripts X and Y denote significant differences ($P < 0.05$) between diets at each segment (comparison across rows); superscripts A, B, C and D denote significant differences ($P < 0.05$) within a diet between segments (comparison across columns). Values that do not share a superscript are significantly different. The detection limit for *Clostridium* Cluster I*, C. perfringens* α toxin I, LT, STa, STb, *E. coli*, K99, F41, F18, F6 and K88 fimbriae was 4 log10 gene copies/g of feces (wet weight).

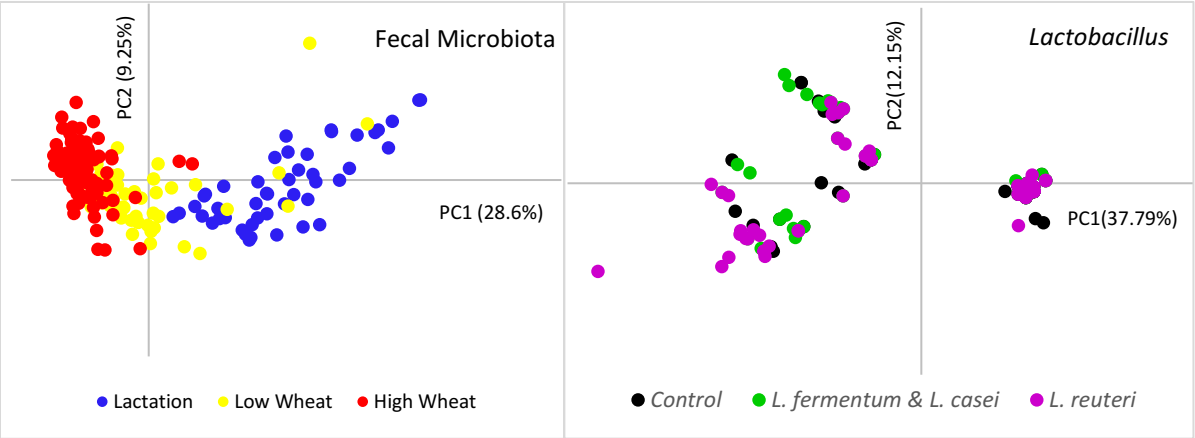
Table S5.3. Gene copy number for *Clostridium* Cluster I, *C. perfringens* α toxin I, LT, STa, STb, *E. coli*, K99, F41, F18, F6 and K88 fimbriae in feces collected on days 0, 7, 14, and 21 [a]

Bacterium or bacterial toxin and time (day)	Log (gene copy no./g) for the following diet:					
	Control	Acidified control	*L. casei / L. fermentum* freeze-dried	*L. casei / L. fermentum*	*L. reuteri* TMW1.656	*L. reuteri* TMW 1.656 Δ*rtcN*
***Clostridium* Cluster I**						
0	10.08±0.18^{A}	9.80±0.45^{A}	9.82±0.17^{A}	9.86±0.23^{A}	9.86±0.26^{A}	10.07±0.13^{A}
7	9.68±0.22^{B}	9.67±0.51$^{A, B}$	9.54±0.29^{B}	9.28±0.57^{B}	9.54±0.47^{B}	9.43±0.33$^{A, B}$
14	9.51±0.45$^{B, C}$	9.35±0.49$^{A, B}$	9.25±0.32^{C}	9.47±0.49$^{A, B}$	9.34±0.25$^{B, C}$	9.24±0.44$^{A, B}$
21	9.32±0.24$^{X, C}$	9.27±0.37$^{X, B}$	8.87±0.18$^{Y, D}$	9.06±0.47$^{X, Y, B}$	9.08±0.23$^{X, Y, C}$	8.80±0.32$^{Y, B}$
***C. perfringens* α toxin**						
0	6.84±0.40^{A}	7.05±0.55^{A,}	6.49±0.35^{A,}	6.65±0.70^{A}	6.71±0.65^{A}	6.26±1.18^{Y}
7	5.96±0.68$^{A, B}$	6.49±1.51$^{A, B}$	5.96±0.92^{A}	6.48±1.47^{A}	6.27±2.34^{A}	5.55±0.32
14	5.13±0.90^{B}	5.00±1.27^{C}	5.34±1.01^{B}	4.59±1.01^{B}	4.56±1.01^{B}	4.59±1.20
21	5.36±1.20^{B}	5.66±1.24$^{B, C}$	5.57±1.29$^{A, B}$	5.19±1.30^{B}	5.73±0.89$^{A, B}$	5.61±0.92
LT						
0	5.63±0.70^{A}	4.81±1.03	5.19±0.53^{A}	5.26±0.80^{A}	5.07±0.67^{A}	5.22±0.77
7	4.98±0.45^{A}	4.90±0.63	4.62±0.78$^{A, B}$	4.71±0.88$^{A, B}$	5.05±0.89^{A}	4.88±0.50
14	4.04±0.91^{B}	4.23±0.91	3.85±0.93^{C}	4.06±1.02^{B}	4.14±1.06^{B}	4.14±0.94
21	4.20±0.69^{B}	4.36±0.34	4.10±0.28$^{B, C}$	4.14±0.40^{B}	4.10±0.29^{B}	4.22±0.33
STa						
0	4.65±0.83	4.85±0.79	4.48±0.72	4.64±0.88	5.07±0.51$^{A, B}$	4.72±0.85
7	5.37±1.26	5.17±1.58	4.61±1.47	5.43±1.63	6.13±1.95^{A}	4.93±1.34
14	4.82±1.45	4.90±1.21	4.99±0.99	5.14±1.00	5.08±1.22$^{A, B}$	4.80±1.23
21	4.37±0.98	4.18±1.07	4.50±1.05	5.18±1.12	3.95±1.09^{C}	4.01±1.06
STb						
0	5.70±0.54	5.64±0.41^{A}	5.65±0.60	5.47±0.40	5.47±0.34^{B}	5.84±0.25
7	5.78±0.23	5.91±0.27^{A}	5.68±0.21	5.80±0.33	6.16±1.07^{A}	5.76±0.38
14	5.48±0.33	5.31±0.36^{B}	5.50±0.25	5.54±0.30	5.52±0.28^{B}	5.59±0.24
21	5.67±0.26	5.65±0.15^{A}	5.58±0.24	5.69±0.31	5.49±0.21^{B}	5.49±0.32
E. coli						
0	7.69±0.74^{A}	8.09±0.98^{A}	7.91±0.76^{A}	7.79±0.67^{A}	8.01±0.87^{A}	7.89±1.19^{A}
7	6.89±1.30^{A}	6.99±1.20^{B}	6.48±0.77^{B}	6.70±0.96^{B}	6.72±0.91^{B}	6.60±1.55^{B}
14	5.74±1.03^{B}	5.79±0.93^{C}	5.87±1.42$^{B, C}$	5.87±0.94^{C}	5.98±0.93$^{B, C}$	5.07±0.94^{C}
21	5.75±0.99^{B}	5.31±0.72^{C}	5.38±0.86^{C}	5.62±0.5^{C}	5.18±0.95^{C}	5.66±1.24$^{B, C}$
K99 fimbriae						
0	4.88±1.22^{B}	5.38±1.12	5.14±1.27^{B}	4.97±1.08^{B}	5.16±0.68^{B}	5.45±0.90
7	6.46±0.65^{A}	5.83±1.05	6.34±0.71^{A}	5.64±0.82^{B}	6.16±0.5^{A}	5.32±1.54
14	5.85±1.15^{A}	6.23±0.26	5.87±1.08^{A}	6.36±0.39^{A}	6.30±0.36^{A}	5.65±1.43
21	6.26±0.24^{A}	6.11±1.22	4.77±1.54^{B}	5.93±1.00^{A}	5.77±0.98$^{A, B}$	5.45±1.52
F41 fimbriae						
0	<4	<4	5.24±1.28	4.46±1.27	4.85±1.02	4.61±1.21
7	4.45±1.57	4.12±1.46	4.87±1.83	<4	4.15±1.25	4.57±1.48
14	<4	<4	<4	<4	<4	4.34±1.49
21	<4	4.06±1.13	<4	<4	<4	<4
F18 fimbriae						
0	6.74±0.16^{B}	6.77±0.28^{B}	6.72±0.21^{B}	6.72±0.21^{B}	6.57±0.31^{B}	6.74±0.31
7	7.52±0.80^{A}	7.67±0.97^{A}	7.73±0.91^{A}	7.27±0.66$^{A, B}$	7.55±0.69^{A}	7.44±0.90
14	7.49±0.14^{A}	7.61±0.17^{A}	7.56±0.21^{A}	7.75±0.40^{A}	7.54±0.22^{A}	7.66±0.18
21	7.12±0.31$^{A, B}$	7.48±0.64^{A}	6.98±0.22^{B}	7.16±0.44^{B}	7.18±0.49$^{A, B}$	7.35±0.46
F6 fimbriae						
0	6.08±0.28^{B}	6.02±0.44^{B}	5.97±0.52^{B}	5.79±0.30^{C}	5.87±0.37^{B}	6.02±0.23^{B}
7	6.61±0.51^{A}	6.67±0.63^{A}	6.70±0.60^{A}	6.34±0.42$^{A, B}$	6.54±0.42^{A}	6.41±0.59$^{A, B}$
14	6.24±0.20^{B}	6.40±0.25$^{A, B}$	6.30±0.17$^{A, B}$	6.55±0.36^{A}	6.30±0.21^{A}	6.45±0.24^{A}
21	6.26±0.35$^{A, B}$	6.51±0.45^{A}	5.98±0.22$^{, B}$	6.16±0.30$^{, B}$	6.24±0.45$^{A, B}$	6.28±0.43$^{A, B}$

a Data are presented means ± SD (n=48). Superscripts X and Y denote significant differences ($P < 0.05$) between diets at each time point (comparison across rows); superscripts A, B, C and D denote significant differences ($P <$

0.05) within a diet over time (comparison across columns). Values that do not share a superscript are significantly different. The detection limit for *Clostridium* Cluster I*, C. perfringens* α toxin I, LT, STa, STb, *E. coli*, K99, F41, F18, F6 and K88 fimbriae was 4 log10 gene copies/g of feces (wet weight).

Figure S5.1. Fecal microbiota (left) and *Lactobacillus* (right) community structure as determined by Principle coordinates analysis (PCoA) of partial 16S rRNA sequences. Each dot represents individual fecal samples, colored according to the feed phase (left) and according to the wheat content of feed (right).



Chapter 6. Metagenomic reconstructions of gut microbial metabolism in weanling pigs

6.1. Introduction

Culture dependent and culture-independent approaches have advanced our understanding of the assembly of intestinal microbiota and their importance for their host [288–291]. In general, the development of gut microbiota is influenced by host genetic variation [289,292,293], environmental factors and stochastic events [292,294,295]. The association between the host genetics and gut microbiome is mediated by immunity-related pathways and the secretion of antimicrobial compounds [289]. Multiple environmental factors, such as antibiotics, social contacts and the environment also shape the architecture of gut microbiota [292,294,296]. The diet and particularly dietary carbohydrates are a key determinant for the composition and activity of the intestinal microbiome.

In mammals, lactose is initially the major or the only dietary carbohydrate. In general, mammals gradually transition from lactose to plant carbohydrates as main source of dietary carbohydrates; this dietary shift also induces a major shift of the intestinal microbiome [288,292,294,297,298]. In contrast, current swine production systems impose an abrupt transition from sow's milk to solid food in piglets, this also induces an accelerated succession of microbial communities post-weaning [118,294]. In commercial pig production, carbohydrates are the main energy source for pigs, comprising more than 60% of the dry matter and 60-70% of the dietary energy intake [299,300]. Dietary glycans that are not hydrolyzed and absorbed in the upper intestine enter the hindgut; bacterial fermentation to short-chain fatty acids also provides metabolic energy to the host [295]. Because specific microbial taxa are specialized for degradation of specific dietary carbohydrates, the composition of the diet alters the composition and activity of intestinal microbiota [300].

Consequently, gut microbial communities play a pivotal role in facilitating better adaption of piglets to fibrous feed and in minimizing the risk of colonization by pathogens after weaning [301]. Current analyses of intestinal microbiota are largely based on sequence-based methodology, avoiding the time-consuming culture-based analysis of intestinal microbiota. When assessing the function of intestinal microbiota on the basis of high-throughput sequencing data, metagenomic binning and genome-scale metabolic reconstructions has bridged the gap between the taxonomic analysis of microbial communities on the basis of 16S rRNA sequences and the description of the metabolic repertoire of individual members of gut microbiome by analysis of the abundance and distribution of metabolic enzymes [302]. Metagenomic binning is also an essential tool to understand metabolic cooperativity of between different representatives of the microbiome. Metabolic binning of metagenomic sequence data and the assignment of bacterial taxonomy to metabolic activity is thus an important tool to substitute untargeted microbiome modulation with targeted or predictable modulation of gut microbiome [303].

Swine are an important livestock species. Particularly at the time of weaning, dietary management of the piglets' microbiome is an important tool to reduce the piglets' susceptibility to pathogens, and to improve feed efficiency. However, past studies of the interactions between gut microbiome and diets in pigs were limited to 16S rRNA gene sequencing, or metagenomic analyses without metagenomic binning [23,294,304]. This study therefore aimed to unravel the adaptation of the swine microbiome to the dietary shift after weaning, and to establish a metagenomic reference by binning of genomes of swine gut bacteria from 72 samples from 18 animals. The metagenomic reference was used to predict the metabolic capacity of the fecal microbiome for metabolism of dietary carbohydrates by CAZy annotation, and by detailed analysis of metabolic pathways of major substrates present in wheat.

6.2. Materials and methods

6.2.1. Diets, animals and samples

Six experimental diets were prepared by mixing 98% basal diet (Table S6.1) with 2% unfermented wheat, acidified wheat (pH 3.8), wheat fermented with *L. casei* K9-1 and *L. fermentum* K9-2 (CanBiocin Inc, Edmonton, AB, Canada), or unfermented wheat with freeze-dried cultures of *L. casei* K9-1 and *L. fermentum* K9-2 (approximately 10^9 CFU /g), wheat fermented with *L. reuteri* TMW1.656 and wheat fermented *L. reuteri* TMW1.656Δ*rtcN* (Table S6.2) [267]. Feed fermentation was performed as previously described [13, 20].

The six dietary treatments were randomly allocated to 48 crossbred castrated male piglets (21 days of age) with randomized block design to provide 8 replicates per dietary treatment. Pigs were raised in a temperature-controlled room (28 ± 2.5^{o}C) with one pig per pen and divided into six blocks. Pigs had access to *ad libitum* feed and clean water.

A total of 191 fecal samples were collected from the pen floors of at days 0, 7, 14 and 21. The fecal samples were immediately stored at -20^{o}C after sampling. Subsamples samples (2-3 g) were stored at -80^{o}C after thawing and mixing the frozen samples. Total bacterial DNA was extracted from fecal samples using QIAamp Fast DNA stool mini kit (Qiagen, Inc., Valencia, CA, USA) based on the manufacture's protocol. Purified DNA with an A260/280 ratio higher than 1.8 were selected for further analysis.

6.2.2. Intestinal microbial community analysis using 16S rRNA gene sequencing

Genomic DNA was sequenced on Illumina MiSeq (2 × 300 bp reads) by amplifying the V5-V6 domain of the 16S rRNA gene (University of Minnesota Genomics Center, Minneapolis, MN, USA). A total of 6,647,893 sequences with an average length of 266 bp, corresponding to 34,805 16S rRNA sequences for each of the 191 samples, were retained for downstream analysis after the

quality filtering from QIIME pipeline (MacQIIME 1.9.1-20150604) [233]. Sequences with 97% similarity were clustered into Operational Taxonomic Units (OTUs) by UCLUST [234] after de-replication and de-multiplexing. The GreenGenes database was used for taxonomy assignment with 95% average nucleotide identity (ANI) at the genus level. OTUs that were represented by only one or two sequences (relative abundance < 0.005%) were discarded. Principle coordinates analysis (PCoA) and analysis of similarities (ANOSIM) were performed using weighted UniFrac distance matrix calculated by *beta_diversity.py* [294].

6.2.3. Metagenomic sequencing, assembly, binning and genome annotation

Samples from 18 pigs taken at 0, 7, 14, and 21 d after weaning were selected for shotgun metagenomic sequencing. Sequencing were performed on Illumina Hiseq 2500 PE125 platform with low input library protocol (McGill University and Genome Quebec Innovation Center, Montreal, Canada). A total of 399.25 Gb of sequence data were obtained, corresponding to 2.22 x 10^{11} reads for each of the 72 samples. After quality check by FastQC, adapters were trimmed from raw reads by Trimmomatic [305] using a local adapter database. Trimmed reads were assembled into contigs using IDBA_UD with default parameters.

Binning was performed with MaxBin2 [306] using contigs longer than 3000 bp. After a two-step de-replication with dRep [307], 596 bins were obtained from the sample clusters pooled by pigs (four time points for each pig). CheckM [308] assessment indicated that all 596 bins were ≥ 50% complete, 458 were substantially complete (completeness ≥ 70%), 240 were nearly complete (completeness ≥ 90%) [309] (Table S6.3). Of 458 substantially complete bins, 360 bins with contamination < 5% were regarded as high-quality assembled genomes and selected for further analyses.

Open reading frames (ORFs) were identified by prodigal v.2.6.1[310]. ORFs were annotated with BLAST against Clusters of Orthologous Groups (COG) database and CAZy database with an e-value ≤ 1e-5 [311].

6.2.4. Phylogenetic identification and calculation of the relative abundance

The taxonomy of 360 high quality bins was assigned by *Phylophlan* with 3,737 reference genomes [312] on the basis of 400 proteins. The bins were assigned at the species, genus and family level when average amino acid identity of encoded proteins to the reference genome was greater than ≥ 90%, 60% and 45%, respectively [313,314], in at least 50 proteins [315]. The average coverage of bins were determined using MaxBin2 [306] by recruiting reads (from each sample) to scaffolds. The average coverage normalized to the total number of reads in each sample corresponds to the relative abundances of bins.

6.2.5. Reconstruction of metabolic pathways for carbohydrate fermentation

CAZy were clustered into 5 categories based on the substrate specificity of glycoside hydrolases (GHs) and carbohydrate esterases (CEs). Enzymes from the GH families GH13, GH31, GH97, GH4, GH14, GH15, GH57 and GH63 were assigned to starch degrading enzymes. GH families GH32, GH91 and GH68 were assigned as fructan hydrolysing enzymes. GH families containing β-glucanases including licheninase, β-glucan endohydrolase, endo-(1, 4) β-glucanase are GH8, GH16, GH26, GH5, GH6, GH9, GH10, GH12, GH44, GH48, GH45, GH51. GH an CE families harboring xylanase, arabinofuranosidase, α-glucuronsidase and acetyl-xylan esterase were regarded as arabinoxylan specific and include GH5, GH10, GH11, GH8, GH43, GH51, GH67, GH115, CE1, CE2, CE4, CE6 and CE7. Enzymes degrading O-linked and N-linked host glycans include GH20, GH84, GH110, GH89, GH125, GH109, CE14, GH123 and CE9. The degradation capacity of each bin corresponds to the sum of positive hits of GHs or CEs under each category.

Metabolic pathways of starch, fructan and lactose were studied by blasting sequences of key enzymes that were characterized biochemically (Table S6.4) against 360 assembled genomes. An amino acid identity of ≥ 40% and e-value ≤ 1e-5 were used as threshold values. The relative abundance of enzymes over time was calculated by sum of all positive hits normalizing with corresponding abundance of target bins at four time points.

6.2.6. Statistical analysis

Data analyses were performed in R, (version 3.4.3, The R Foundation for Statistical Computing, https://www.r-project.org/). *P* values < 0.05 with Bonferroni-adjustment were considered significant. Results are presented as means ± standard deviation. The average daily gain, feed intake of pigs, feed efficiency and relative abundance of bin were compared using linear mixed-effects (LME) models based on randomized complete block design with repeated measurement. In the model, pigs were considered as experimental unit, time and block were considered as fixed effects and random effect, respectively. Alpha-diversity parameters between time points were compared using Kruskal-Wallis rank-sum test. The UniFrac distance of pig groups were also compared using LME models with time*sow source as fixed effects.

6.3. Results

6.3.1. Growth performance and gut health of pigs

The grow performance of pigs in 21 days after weanling is listed in Table 6.1. Both average feed intake and average daily gain increased throughout the experimental period. The feed efficiency increased in the first two weeks and plateaued in the last week. All animals remained healthy during the 21-day trial.

Table 6.1. Growth performance of weanling pigs during the first 3 weeks after weanling

Time	Day 7	Day 14	Day 21
Average feed intake [a] (g DM [b] /day)	267 ±2.90 [C]	469±4.11 [B]	749±16.85 [A]
Average daily gain (g / day)	177±11.48 [C]	375±11.33 [B]	615±17.49 [A]
Feed efficiency (G / F)	0.65±0.04 [B]	0.80±0.02 [A]	0.84±0.03 [A]

Data was presented as mean ± standard error of means. Results with unlike letter in the same row were significant different ($P < 0.05$).

[a]Pigs were fed with phase 1 diet (80% basal diet + 20% wheat flour) for the first 7 days, followed by phase 2 diet (50% basal diet + 50% wheat flour) from days 8 to 21.

[b]DM, dry matter.

6.3.2. Bacterial community composition analysis by 16S rRNA gene sequencing

Analyses of the microbiome composition determined factors that influence the evolution of the microbiome after weaning (Figure 6.1 & Figure S6.1). Alpha diversity increased after weaning and remained stable after week 3 (Figure 6.1). The presence of probiotic lactobacilli in the diet [267] did not influence the composition of fecal microbiota. Significant but minor differences were observed between individual animals (Table 6.2). Litter effects were significant at weaning but not at later sampling times (Figure. 6.1). The differences between bacterial communities were mainly explained by wheat content of the diet and the time after weaning (Table 6.2). The effects of time and wheat inclusion on bacterial composition were visualized by PCoA based on weighted UniFrac distance matrix (Figure S6.1). PCoA clearly grouped samples based on time after weaning and wheat content (Figure S6.1). Therefore, subsequent analyses focused on microbial degradation of carbohydrates.

Table 6.2. ANOSIM[a] of weighted UniFrac a distance matrix calculated with partial 16S rRNA sequences

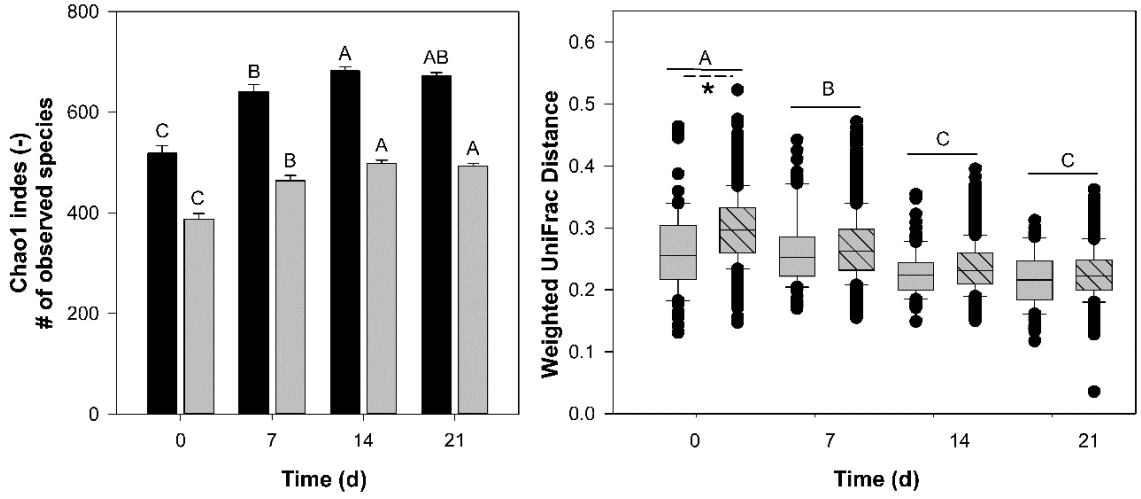
Factors	UniFrac distance (weighted)		
	N	R[b]	*p*
Probiotic	6	0.008	0.122
Animal	48	0.087	0.001
Sow	11	0.100	0.001
Age	4	0.332	0.001
Wheat	3	0.505	0.001

[a]ANOSIM, Analysis of similarity.

[b]Slight correlation was considered when $0 < R < 0.3$, whereas $R > 0.3$ was considered a strong correlation.

Figure 6.1a. α-Diversity of fecal microbiota over time. Black bars represent Chao1 indexes, grey bars represent the number of observed species in each sample. Data were calculated from partial 16S rRNA sequences and are presented as mean ± standard errors of the means (n = 48). Mean values for the same index (bars with same color) with unlike letters or asterisk (*) are significantly different ($P < 0.05$).

Figure 6.1b. UniFrac distance (weighted) between fecal microbiota of piglets from the same sow (gray bars) and from all piglets (hatched bars) during the first 3 weeks after weanling. Mean values for the same group (bars with same color) and pairs at the same time point with unlike letters are significantly different ($P < 0.05$).



6.3.3. Metagenomic reconstruction of fecal microbiome in weanling pigs

Reconstruction of bacterial genomes from metagenomic sequence data generated a total of 596 genomic bins from 18 weanling pigs and enabled a genome-based investigation of microbial metabolism. The average size of 598 bins was 2.07 Mb and the average N50 was 32,152 bp. Figure 6.2 shows the taxonomic identification of 360 bins with completeness of > 70% and contamination of < 5%). Of the 360 identified bins, 216 were assigned to *Firmicutes* and 96 to *Bacteroidetes*; 11 bins were identified as *Actinobacteria*, 16 as *Proteobacteria*. Only 106 of the metagenomics bins were identified at the genus or species level; remaining bins did not match to genome-sequenced reference strains. The relative abundance of bacterial genomes was calculated based on the average coverage per metagenomics bin normalized to the number of total reads in each sample (Figure 6.2, Table S6.3). About half of the bins (175 of 360) showed differences in abundance over time. Among these, 56 bins showed a higher abundance at 0 d when compared to other sampling times while the abundance of 64 bins increased over time. One of the bins with decreasing abundance represents *Lactobacillus delbrueckii*, which has to date not been considered a representative of animal intestinal microbiota. Interestingly, 20 bins only increased temporarily at day 7 and / or day 14.

The capacity of 360 bins for glycan degradation was initially predicted by identification of GHs and carbohydrate esterases with similar substrate preference (Figure 6.2, Table S6.3). Starch-degrading enzymes were widely found in genomes of Firmicutes and *Bacteroidetes*. These metagenomic bins were relatively abundant and increased over time, particularly in genomes of *Faecalibacterium* spp. Only few genomes harboured fructan degrading enzymes, examples include genomes of *Lactobacillus, Escherichia coli* and several unclassified *Bacteroidales*. The distribution of bins carrying enzymes for β-glucan, arabinoxylan and host-glycan metabolism

overlapped; these GH families were widely distributed in genomes of *Bacteroidetes*, *Ruminococcus* and *Lachnospiraceae*.

6.3.4. Reconstruction of metabolic pathways for starch, fructan and lactose metabolism in weanling pigs

Microbial metabolism of starch, fructan and lactose was further analysed by identification of metabolic enzymes degrading starch, fructans, and lactose [316–323] (Figure 6.3). Query sequences were selected to retrieve all characterized metabolic pathways for the substrate with minimal overlap between hits obtained with different query sequences for the same substrate.

Firmicutes and *Bacteroidetes* harboured distinct pathways for starch degradation. *Firmicutes* convert starch by an extracellular α-(1→4)-glucan branching enzyme (GlgB) and pullulanses (Amy12), these enzymes occurred only in *Firmicutes.* GlgB was detected in 191 of 216 bins assigned to *Firmicutes*. Remarkably, most of the starch-degrading *Firmicutes* carry GlgB but no other starch hydrolysing enzymes; only 5 members of *Faecalibacterium prausnitzii* and one *Eubacterium rectale* additional carry pullulanases. Bin 254 was the only member of *Firmicutes* with an extracellular neopullulanse2. The periplasmic starch utilization by *susA* (GH13, 54.2%) and *susB* (GH97, 74.0%) was present in *Bacteroidetes*. Multiple metagenomics bins assigned to *Bacteroidetes* contained multiple genes for starch digestion, including extracellular and periplasmic enzymes. Among the 80 starch-degrading *Bacteroidetes*, only two *Bacteroidetes* were without *susA* or *susB*, 23 *Bacteroidetes* additionally harboured extracellular amylase (Amy1, 4) or neopullulanse (SusG).

Firmicutes and *Bacteroidetes* also harboured distinct enzymes for fructan hydrolysis. *Firmicutes* catabolize fructan by intracellular β-fructofuranosidase (ScrA / ScrB, GH32) and extracellular fructansucrases (Inu, GH68, and FruA, GH32). FruA, ScrB and Inu were present only in

lactobacilli. ScrA was present in other *Firmicutes*, including *Subdoligranulum variabile, Faecalibacterium prausnitzii, Eubacterium* and unclassified *Clostridiales*. *Bacteroidetes* metabolize fructan by β-(2→6) endo-fructanases (GH32) including BT_1760 (extracellular), BT_3082 (periplasmic) and BT_1765/1754 (intracellular). Among 49 fructan-degrading *Bacteroidetes,* only 3 members did not carry intracellular β-(2→6) endo-fructanase, 9 members of *Bacteroidetes* additional harboured extracellular BT_1760, another 2 members additional carried periplasmic BT_3082.

Lactose hydrolysis was identified only in *Firmicutes* with exception of bin20 representing a member of *Coriobacteriaceae*. Most lactose-degrading bacteria (27 out of 31) hydrolyze lactose by intracellular GH2 β-galactosidase (BbgI, LacM and BbgIV) or GH42 β-galactosidase LacA, including members of *Lactobacillus, Subdoligranulum, Ruminococcus.* The abundance of *Lactobacillus delbrueckii*, a species that is specialized for lactose conversion, decreased over time (Figure 6.2). The other lactose decomposers were capable of lactose hydrolysis by extracellular BbgIII (GH2).

Figure 6.2. Phylogeny, abundance, and metabolic potential of bacterial taxa in the fecal microbiota of piglets. Bacterial taxa were identified based on reconstructed genomes assigned to 360 bins with ≥ 70% completeness and < 5% contamination. The phylogenetic tree and the taxonomic assignment of reconstructed bins are shown as the innermost layers. The taxonomic assignment was based on the average amino acid identity of encoded proteins to the most closely related reference genome sequence. Branches and labels with different colors represent different phyla as indicated by the color code to the lower left. The heatmap in the third layer depicts the relative abundance of the 360 bins, inside to outside 0, 7, 14, and 21 d (n = 18 per time point). The relative abundance of bins in each sample was calculated from the average contig coverage obtained by re-mapping reads form samples and normalizing to the total reads in the sample. The outermost four layers depict the number of glycosyl hydrolases and esterases encoded in each bin. Glycosyl hydrolases and esterases were grouped by their predicted substrate specificity as follows: Lactose degrading enzymes include GH1,GH2 and GH42; Starch degrading enzymes include GH13, GH31, GH97, GH4, GH14, GH15, GH57 and GH63; fructan degrading enzymes include GH32, GH91 and GH68; β-glucan degrading enzymes include GH8, GH16, GH26, GH5, GH6, GH9, GH10, GH12, GH44, GH48, GH45, and GH51; arabinoxylan degrading enzymes include GH5, GH10, GH11, GH8, GH43, GH51, GH67, GH115, CE1, CE2, CE4, CE6 and CE7; host-glycan degrading enzymes include GH20, GH84, GH110, GH89, GH125, GH109, CE14, GH123 and CE9.

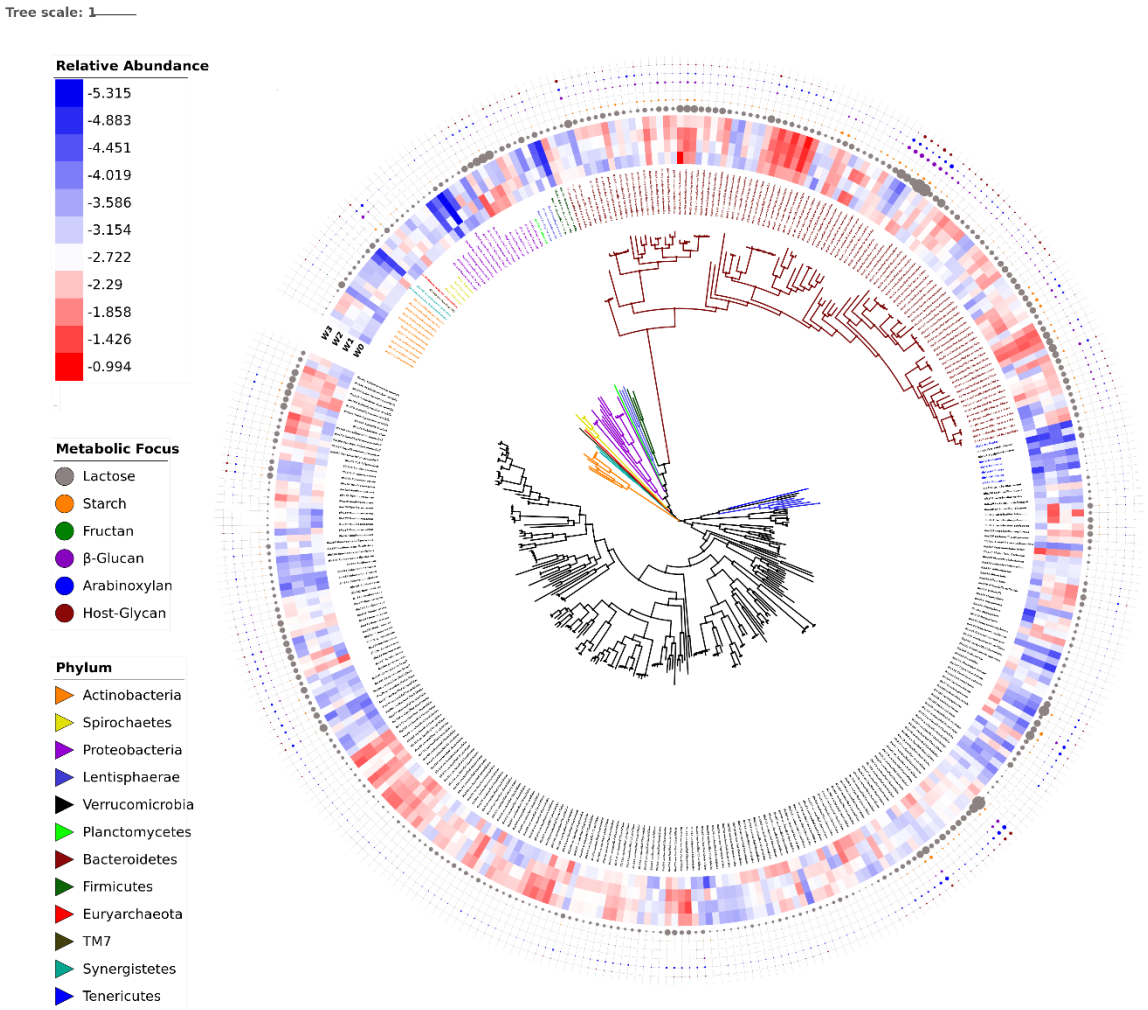
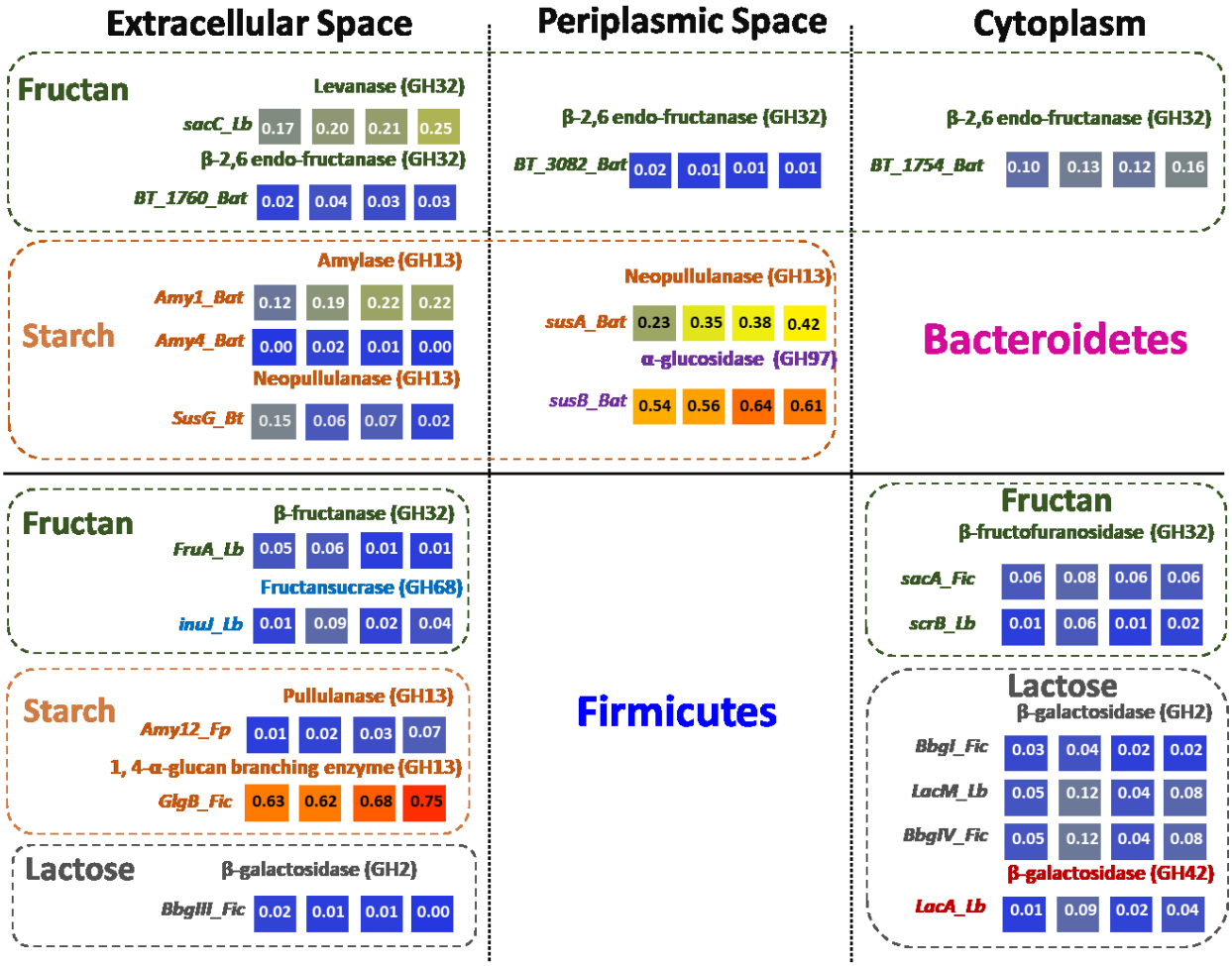


Figure 6.3. Predicted metabolic pathways for starch, fructan, and lactose metabolism. The abundance of metabolic enzymes was obtained by using biochemically characterized enzymes as query sequences for BLAST analysis of metagenomics bins. Enzymes are grouped by the substrate and the cellular location of the query sequence. The abundance of corresponding genes at the four time points was calculated from the cumulative relative abundance of bins encoding for a homologue of the gene and is shown as colour coded matrix for the four time points (left to right 0, 7, 14, and 21 d). Labels at the left side for rows include the name of gene and the abbreviations for the organism for which the corresponding enzyme was characterized. Abbreviations for organisms are as follows: *Bat, Bacteroidetes; Bt, Bacteroides thetaiotaomicron; Lb, Lactobacillus; Fp, Faecalibacterium prausnitzii; Fic, Firmicutes.* The accession number of query sequences and reference to the biochemical characterization of the enzymes is provided in Table S6.4 of the online supplementary material.



6.4. Discussion

6.4.1. Microbial composition in piglets differed by host derived factors and reshaped by diet

The fecal microbiota of weanling pigs is unstable during early life and stabilizes within 2-3 weeks after weaning [118,304]. In this study, we analyzed the structure and function of the intestinal microbial community in piglets during the first three weeks after weaning by partial 16S rRNA gene sequencing and metagenomics analysis. Diet together with age were the most significant factors shaping community assembly in weaning piglets; litter effects were transient and minor. Age alters the physiology of the gastrointestinal tract including immune and metabolic functions during weaning in piglets, these changes occur rapidly in response to the transition to solid food [256,324].

Litter effects in the structure of fecal communities were significant at weaning and the diversity of animals from different sows was higher than the diversity of littermates. Initial differences in microbiota structure between animals and litters were altered by the uniform post-weaning diet. Inclusion of probiotic bacteria gastric communities of lactobacilli [267] but did not significantly alter the structure of fecal microbiota. The use of the same probiotic *L. reuteri* at a 10-fold higher dose significantly altered the abundance of only very few bacterial taxa [23]. The limited impact of probiotic bacteria on the overall composition of intestinal microbiota matches observations in humans [256,325].

6.4.2. Novel reference with 596 genomes were reconstructed for swine fecal microorganisms

The importance of the intestinal microbiome for host physiology highlights the need for comprehensive analysis based on genomic and phenotypic assays. Culture-dependent analysis of intestinal microbiota, however, lags the identification of bacterial taxa by high-throughput

sequencing approaches [290,326]. This study reconstructed 596 genomes including 360 high quality and substantially completed genomes from swine fecal microbiota. Owing to the high error rates in sequencing and assembly of regions with repetitive sequences including rRNA operons [327], bacterial taxa were identified on the basis of the AAI of multiple conserved proteins sequences distributed across the genomes [313]. Only 106 genomes were assigned to the genus or species level due to the lack of matching genome sequences of cultured species. For the remaining 254 high quality genomes, this study provides novel genome sequence data and expands current knowledge on the phylogenetic and metabolic diversity of swine intestinal microorganisms [328,329]. The phylogenetic analysis of 360 reconstructed genomes and the annotation of open reading frames serves as the first reference for metagenomes of swine microbiota. Different from past swine metagenomics studies [294,304,330] this reference allows the assignment of the metabolic activity of intestinal organisms to their taxonomic identification.

6.4.3. Phylogeny and functions of high-quality genomes reconstructed for swine fecal microorganisms

The increasing abundance of *Bacteroidales* and *Clostridiales* over time reflects their ability to derive metabolic energy from diverse plant polysaccharides [321,331–333]. CAZy annotation indicated the ability of multiple *Bacteroidetes* species to degrade starch, β-glucan, arabinoxylan and host glycans [317]. *Ruminococcaceae,* a family in the phylum *Firmicutes*, also includes species with the capability to hydrolyse a broad range of polysaccharides, matching the identification of *R. bromii* as a keystone species for starch degradation in the human colon [334]. The relative abundance of metagenomic bins representing bacterial taxa with multiple polysaccharide degrading enzymes increased in response to the inclusion of plant carbohydrates after weaning. Examples include members of *Bacteroidetes* and *Ruminococcaceae*. In contrast,

some early colonizers, mostly *Proteobacteria* including *E. coli* and members of the genus *Clostridum* decreased dramatically after weaning. The reduced abundance of *Proteobacteria* may relate to a lower protein intake concomitant with a higher fiber intake and a more developed immune function [324,335]. Remarkably, metagenomics binning also identified *Lactobacillus delbrueckii* in the microbiome of piglets; this organism decreased rapidly after weaning. *L. delbrueckii* has not been considered a member of animal intestinal microbiota [131]. Different from other species of the *L. delbrueckii* group, which maintain enzymes for metabolism of a relatively broad array of carbohydrates [131], the genome of *L. delbrueckii* underwent reductive evolution that silenced most carbohydrate metabolic enzymes [336]. This metabolic focus of *L. delbrueckii* on lactose as main source of metabolic energy was interpreted as adaptation to the milk environment or dairy fermentations [337], however, our data suggests that the metabolic focus on lactose may alternatively represent adaptation to the intestine of suckling mammals. Re-analysis of the intestinal microbiome of weanling piglets [23] indeed revealed that *L. delbrueckii* was also detected in piglets on the day of weaning but no longer detectable 2 or 3 weeks after weaning (Figure S6.2).

6.4.4. Microbial degradation of starch, fructans and lactose in weanling pigs

Even though a large panel of CAZymes have been catalogued based on substrate specificity, CAZy-family based classification of enzymes needs to be complemented by a more detailed analysis that is based on reference sequences of enzymes that were biochemically characterized [317,338]. Moreover, classification of proteins in GH or CE families not always allows an unambiguous prediction of their substrate specificity or cellular location. Many substrates are degraded by enzymes several from several families, and enzymes in many GH or CE families are active on more than one substrate [331].

In humans, starch entering the large intestine is degraded by microbial consortia contributing diverse extracellular, periplasmatic and intracellular starch-converting and –hydrolysing enzymes [339] while lactose, GOS and dietary fructans are degraded by few bacterial groups, particularly *Bifidobacterium* spp. [340,341]. Our analysis revealed that microbial consortia and species that degrade starch, fructans, and lactose in weaning piglets differ substantially from those that were identified in humans.

Bacterial degradation of starch is mediated by amylases and pullulanases, which hydrolyse α-(1→4)- and α-(1→6)-glucosidic bonds, respectively [319]. Members from GH13 families were identified as the principal starch degrading enzymes but *Firmicutes* and *Bacteroidetes* used distinct starch-utilization systems. Extracellular glycosidases were identified mainly in *Firmicutes* while periplasmic enzymes were only found in *Bacteriodetes*. The high abundance of an extracellular α-(1→4)- glucan branching enzyme suggests this enzyme is important for the primary degradation of starch in the swine GIT. The α-(1→4)-glucanotransferase GlgB catalyses glucan chain transfer to form α-(1→6)-glucosidic linkages; this enzyme was found in *Firmicutes* only. The enzyme was suggested to improve accessibility to insoluble starch [320,342] and is broadly distributed in intestinal microbiota of different hosts including humans, chicken, cattle, and swine [343]. Following starch hydrolysis by extracellular enzymes, the α-glucosidases SusA and SusB further degrade gluco-oligosaccharides in the periplasm. Disruption of SusA and SusB from *B. thetaiotaomicron,* reduced the rate of growth but did not eliminate the growth of the strain [344]; however, periplasmic starch degrading enzymes may reduce access of competitors to the products of hydrolysis. With exception of *F. prausnitzii*, starch degrading *Firmicutes* carried a single glycosidase; in contrast, redundant enzymes with different activities or different locations were commonly detected within a single genome of *Bacteroidetes*. The high abundance of starch

utilizing enzymes in fecal microbiota demonstrates that wheat starch, despite its hydrolysis by pancreatic amylases and brush border enzymes, is a major carbohydrate source for colonic microbiota. The distribution of extracellular and periplasmic enzymes for starch degradation highlights a high level of metabolic cooperativity that was also noted in human starch and cellulose degrading microbial communities [317,334]. In humans, *Ruminococcus bromii* plays a key role in fermentation of type 3 resistant starch and enhanced the growth of *B. thetaiotaomicron*, *B. adolescentis* or *E. rectale* on resistant starch [322,334], however, this study indicates that this species does not fulfill a comparable role in swine microbiota.

Fructans with an average degree of polymerization of 5 - 6 are among the major non-starch polysaccharides in wheat [345]. Fructans were degraded by *Bacteriodetes* and lactobacilli. The linear structure of fructans allows hydrolysis by single enzyme that are classified in the GH32 or GH68 families [320]. In contrast to the complex and partially redundant starch degrading enzymes in *Bacteriodetes*, fructans degraders carried fewer fructanases. Fructan utilization is not conserved within members of a specific species [39,40,66]. Three GH32 enzymes, including BT1760 (extracellular), BT3082 (periplasmic), and BT1765 (intracellular), as well as hybrid two-component (HTC) signaling system, BT1754 are required for fructan utilization in *B. thetaiotaomicron* and related *Bacteroides* spp.[321]. *B. thetaiotaomicron* utilized levan while *B. caccae* ferments inulin [321,345]. GH 68 family enzymes in lactobacilli are extracellular levansucrases which are necessary for biofilm formation on non-secretary epithelia of the upper GI tract; these enzymes synthezise levan but do not contribute to fructan hydrolysis [347]. Intracellular GH32 β-fructofuranosidases of lactobacilli (scrB) utilize only fructans with a DP of less than 4 that are transported across the membrane [316,346,348]. The metagenomic analysis is the first to report the presence of extracellular fructanases in intestinal lactobacilli. FruA is

common in oral streptococci, however, its presence is exceptional in lactobacilli and was previously identified only in type II sourdough microbiota [323]; The exclusive presence of FruA in *Lactobacillus* species representing swine intestinal communities may reflect specific nutritional requirements in pigs. The identification of *Bacteriodes* and *Lactobacillus* spp. as major degrading organisms also contrasts microbial consortia degrading fructans in the humans intestine, where bifidobacteria are the main organisms that degrade fructans and benefit from fructan addition to the diet [349].

Lactose accounts for about 26.7% of sow milk solids [350]; transition diets contain 10-15% of lactose. Lactose is thus one of the major dietary carbohydrates in suckling and weanling pigs; wheat contains only low quantities of polygalactans[323]. Only *Firmicutes* fermented lactose with the LacS/LacLM pathway widely distributed in lactobacilli [351–353]. Lactose is transported into the cytoplasm by lactose permease and hydrolysed by intracellular GH2 β-galactosidases common in *Firmicutes*, and GH42 β-galactosidases of lactobacilli. Lactobacilli colonize the stomach of swine where dietary lactose is available; in contrast, *Bacteroidetes* are dominant only in hindgut microbiota after full or partial digestion of lactose in the small intestine. Accordingly, their extracellular enzymes may target β-glycosidic linkages in host or plant glycans rather than lactose. Microbial fermentation of lactose in the terminal ileum and the large intestine contributes to lactose digestion particularly in lactase-non-persistent humans [354]. The distribution of β-galactosidases in human microbiota remains poorly characterized but bifidobacteria are considered to be the main organisms involved in metabolism of lactose and related β-galacto-oligosaccharides [341].

In conclusion, we present a metagenomics reference for swine intestinal microbiome by assigning taxonomies and metabolic functions to the 360 high quality assembled genomes. Along with the clear evidence for dietary carbohydrates acting as the most significant drivers for diversification

of microbiota, we further determined the key microbial contributors to degradation of major substrates in starter diet, including starch, fructans, and lactose. Starch is a substrate for colonic microbiota and its metabolism is dependent on metabolic co-operativity between *Firmicutes* and *Bacteroidetes*. Fructans and lactose are fermented by simple enzyme systems present in *Bacteroides* and *Lactobacillus* spp., respectively. This study greatly improved the functional analysis of swine intestinal microbiome. It also enables future studies linking composition and function of piglet microbiota to establish feeding systems that improve feed efficiency and animal health while reducing microbial resistance to antibiotics.

Supplementary materials

Table S6.1. Composition of diets to fulfil NRC recommendations (2012) for pigs (5-11kg)

Ingredients (%)	Phase 1 (day 0 to 6)	Phase 2 (day 7 to 21)
Wheat HRS (NRC)	20.00	50.00
Corn (NRC)	31.54	1.76
Lactose	15.00	10.00
Soybean meal (NRC)	15.00	15.00
B. napus canola meal		2.50
Soy protein conc HP300	3.00	2.50
Herring meal	6.00	2.50
Corn DDGS [a] (NRC)		5.00
Canola oil (NRC)	4.00	3.40
Limestone	1.15	1.10
Salt	0.50	0.50
Other vitamin and mineral ingredients	3.31	5.24
TiO2	0.50	0.50
Total	100	100

[a] DDGS, distiller's dried grains with soluble

Table S6.2. Experimental design and diets used in this study

Diet	Group	Components	*Lactobacilli* strains
Unfermented diet	Control	basal diet + 2% unfermented wheat	N/A
	Acidified Control	basal diet + 2% unfermented wheat + lactic and acetic acids	N/A
	Freeze dried Canbiocin probiotics	basal diet + 2% unfermented wheat + freeze dried Canbiocin probiotics	*L. fermentum* and *L. casei*
Fermented diet	Canbiocin probiotics	basal diet + 2% fermented wheat with Canbiocin probiotics	*L. fermentum* and *L. casei*
	Reutericyclin	basal diet + 2% fermented wheat with reutericyclin producing *Lactobacilli*	*L. reuteri* TMW1.656
	Non-reutericyclin	basal diet + 2% fermented wheat with non-reutericyclin producing *Lactobacilli* mutant	*L. reuteri* TMW1.656Δrtc N

Figure S6.1. Principle coordinates analysis (PCoA) of fecal microbiota composition based on weighted UniFrac-distance of partial 16S rRNA sequences. Each dot represents individual fecal samples, colored according to the sampling age (Panel A) and according to the wheat content of feed (Panel B).

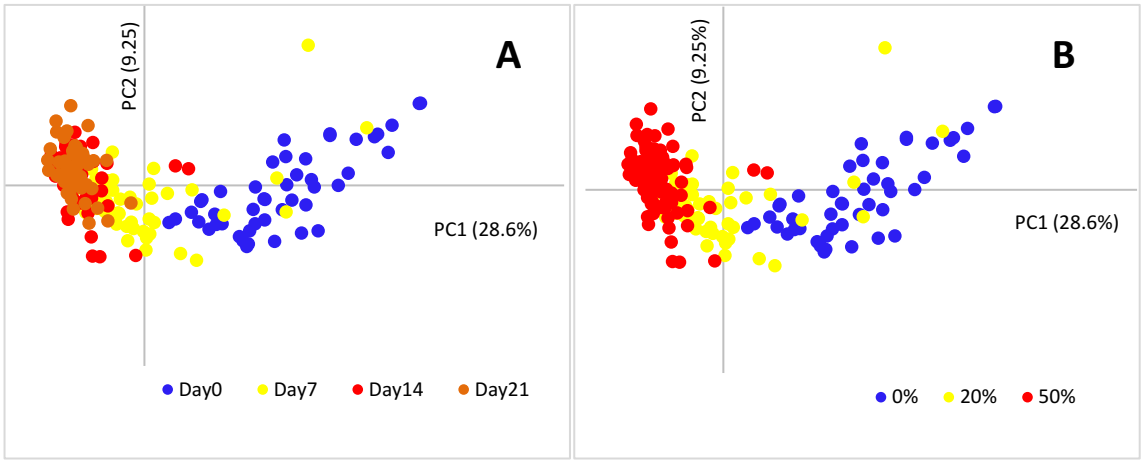


Table S6.3. Quality assessment of 596 bins by CheckM (* Phylogenetic affiliation were only assigned to 360 bins with completeness ≥ 70% and contamination ≤ 5%)

Bin_ID	Phylogeny *	Genome size (bp)	# contigs	N50	GC %	Completeness %	Contamination %
Bin206	unclassified *Clostridiales*	1830849	1827	28008	37.9	93.18	1.34
Bin154	*Ruminococcaceae*	1762869	1672	85982	38.5	85.68	0
Bin196	*Ruminococcus flavefaciens*	2578630	2253	34763	38.5	83.22	0
Bin155	*Ruminococcaceae*	2082788	1954	45052	39.4	95.66	0.34
Bin326	*Phascolarctobacterium*	2057086	2008	25219	38.2	98.2	0.6
Bin156	*Ruminococcaceae*	1655009	1639	15466	38.4	80.98	0
Bin33	unclassified *Bacteroidales*	2244629	1736	18188	36.6	80.59	0.74
Bin207	unclassified *Clostridiales*	1863125	1778	102122	38.4	95.97	0
Bin208	unclassified *Clostridiales*	2436536	2318	102515	36.5	94.92	1.08
Bin157	*Ruminococcaceae*	1554586	1503	66345	35.7	89.93	0.81
Bin34	unclassified *Bacteroidales*	2729792	2247	112084	39.8	95.51	0.48
Bin209	unclassified *Clostridiales*	1698269	1677	17005	36.7	93.66	0
Bin35	unclassified *Bacteroidales*	2297146	2020	24716	37.9	87.3	3.62
Bin3	*Escherichia coli*	4539067	4259	100584	36.2	98.78	0.21
Bin203	*Subdoligranulum variabile*	2419992	2241	54866	36.6	77.55	0
Bin36	unclassified *Bacteroidales*	2271448	1776	17594	38.6	80.49	0.12
Bin197	*Ruminococcus*	2105499	2009	20316	38.6	91.28	3.75
Bin348	*Desulfovibrio piger*	1636123	1660	4524	38.9	70.59	1.78
Bin210	unclassified *Clostridiales*	1732346	1649	82781	38.9	96.15	0
Bin37	unclassified *Bacteroidales*	2342019	2022	12083	36.9	86.11	1.22
Bin211	unclassified *Clostridiales*	1910205	1935	57561	37.8	90.9	0.1

Bin186	*Faecalibacterium*	2360806	2222	116677	37.7	97.54	0
Bin18	*Coriobacteriaceae*	1849683	1765	12665	36.1	91.53	1.61
Bin29	*Bacteroides coprophilus*	2770554	2322	32561	38.3	83.95	1.75
Bin32	*Alistipes putredinis*	3093702	2561	115959	36.9	99.52	0
Bin212	unclassified *Clostridiales*	2412669	2353	53548	36.4	96.37	1.88
Bin2	*Alphaproteobacteria*	1642539	1774	12239	38	95.51	0.06
Bin4	*Escherichia coli*	4045726	4109	9746	36.2	92.63	1.66
Bin213	unclassified *Clostridiales*	1623393	1767	6376	40.1	84.81	2.02
Bin355	*Pyramidobacter piscolens*	3300405	2874	65943	39.8	91.53	0
Bin31	*Bacteroides vulgatus*	4361813	3981	38325	39.1	86.72	4.48
Bin12	*Mycoplasmatales*	1431294	1527	61079	39.9	92.42	3.37
Bin149	*Lachnospiraceae*	2644724	2483	49401	40.7	95.98	0.38
Bin158	*Ruminococcaceae*	2601857	2435	301243	37.4	99.32	0.17
Bin138	*Clostridium scindens*	2885217	2986	48377	41	94.74	0.33
Bin137	*Clostridium bolteae*	4404879	4344	34725	41.7	97.22	3.39
Bin214	unclassified *Clostridiales*	1778909	1761	45079	38.2	79.87	3.69
Bin349	*Desulfovibrio piger*	2582996	2215	102585	38.8	98.7	0.59
Bin358	*Akkermansia muciniphila*	2562750	2279	23613	39	93.82	0
Bin215	unclassified *Clostridiales*	1531569	1629	12145	38.9	92.31	0
Bin359	*Akkermansia.s muciniphila*	2550919	2188	67327	36.5	92.59	1.69
Bin216	unclassified *Clostridiales*	2620615	2567	43124	38.8	95.94	4.44
Bin38	unclassified *Bacteroidales*	1927438	1643	69052	34.7	93.24	0
Bin39	unclassified *Bacteroidales*	2761614	2250	51677	37.2	95.47	3.58
Bin217	unclassified *Clostridiales*	1319117	1287	22659	39.2	87.27	0.81
Bin345	*Delftia*	3345219	3080	114904	33.6	96.84	0.67
Bin159	*Ruminococcaceae*	2341184	2273	50355	39.7	93.06	2.68

Bin193	*Ruminococcus flavefaciens*	2173514	2173	7496	38.3	90.82	1.79
Bin40	unclassified *Bacteroidales*	2701234	2204	32610	37.2	95.66	1.26
Bin218	unclassified *Clostridiales*	1996919	2051	78492	39.4	90.92	0
Bin41	unclassified *Bacteroidales*	2520126	2207	16383	38.6	88.58	3.52
Bin42	unclassified *Bacteroidales*	1842287	1694	14583	38.2	87.36	2.69
Bin357	unclassified *Opitutae*	2171368	1770	58031	34.1	93.92	0
Bin43	unclassified *Bacteroidales*	2226688	1831	12095	38.2	86.38	1.94
Bin219	unclassified *Clostridiales*	2139860	2139	85995	39.6	99.19	1.34
Bin220	unclassified *Clostridiales*	2043147	1972	53303	37.5	99.19	0
Bin13	*Mycoplasmatales*	1336820	1401	75640	39.7	95.51	0
Bin221	unclassified *Clostridiales*	1352857	1312	14366	38.5	90.28	1.4
Bin44	unclassified *Bacteroidales*	2422237	1853	37116	35.2	93.62	0
Bin222	unclassified *Clostridiales*	1119053	1213	7205	39.1	72.77	2.42
Bin45	unclassified *Bacteroidales*	2159755	1922	11314	37.3	87.2	1.07
Bin341	*Lentisphaeraceae*	2106401	2042	6125	39	78.14	3.27
Bin223	unclassified *Clostridiales*	3208704	3096	81878	38.6	96.55	4.89
Bin224	unclassified *Clostridiales*	1428668	1472	10528	39.7	85.41	4.08
Bin147	*Eubacterium rectale*	3016716	2656	46020	40.5	92.89	2.41
Bin225	unclassified *Clostridiales*	1618420	1616	33696	38.2	88.29	0.67
Bin226	unclassified *Clostridiales*	2261741	2296	60580	39.5	94.97	1.8
Bin46	unclassified *Bacteroidales*	2421738	1986	39489	34.9	96.98	0
Bin8	*Treponema*	2598084	2486	63340	39.3	99.3	0.35
Bin160	*Ruminococcaceae*	1524490	1422	44141	39.5	81.88	0
Bin47	unclassified *Bacteroidales*	2351503	1856	64818	35.8	96.71	0
Bin227	unclassified *Clostridiales*	2613503	2498	19220	37.6	92.17	3.91
Bin161	*Ruminococcaceae*	1681403	1700	36129	39.5	89.57	2.68
Bin228	unclassified *Clostridiales*	2188636	2112	109063	36.9	95.97	0.67
Bin229	unclassified *Clostridiales*	2426086	2508	60578	37.8	97.99	0.67
Bin48	unclassified *Bacteroidales*	2941455	2977	6789	37.4	78.3	2.89
Bin230	unclassified *Clostridiales*	2802820	2671	44895	37.7	97.55	0.42

Bin231	unclassified *Clostridiales*	1899796	1591	10670 4	36.9	98.58	0
Bin162	*Ruminococcaceae*	1766263	1634	52448	38.4	88.37	0
Bin232	unclassified *Clostridiales*	2082760	2024	75827	37.3	99.19	0
Bin49	unclassified *Bacteroidales*	2266810	1784	91382	35.5	96.54	0.75
Bin356	*Thermanaerovibrio acidaminovorans*	3327991	2971	74791	39.7	100	0
Bin233	unclassified *Clostridiales*	2396985	2302	32437	37.1	97.32	1.82
Bin50	unclassified *Bacteroidales*	3729088	3509	11025	39.1	90.78	3.04
Bin194	*Ruminococcus flavefaciens*	2237849	2286	7947	38.7	76.29	2.68
Bin51	unclassified *Bacteroidales*	3498672	3124	14590	39.2	87.95	0
Bin234	unclassified *Clostridiales*	1832603	1807	32183	40.3	99.19	2.96
Bin235	unclassified *Clostridiales*	2781264	2787	13508	37	90.43	3.9
Bin236	unclassified *Clostridiales*	1746954	1797	15807	40.4	96.1	2.15
Bin52	unclassified *Bacteroidales*	3119199	2576	38614	39.7	92.05	2.14
Bin237	unclassified *Clostridiales*	2133212	2062	73764	37.2	88.59	0.67
Bin238	unclassified *Clostridiales*	1579536	1545	83397	40.6	90.94	0.67
Bin19	*Coriobacteriaceae*	1804051	1718	13407	36.1	94.96	2.26
Bin53	unclassified *Bacteroidales*	2502981	2063	44065	36	95.48	1.3
Bin54	unclassified *Bacteroidales*	1712893	1628	9816	37.8	75.44	1.07
Bin163	*Ruminococcaceae*	2238204	2226	44483	38.6	96.98	0
Bin327	*Phascolarctobacterium*	2187908	2100	63911	37.8	99.98	0.6
Bin239	unclassified *Clostridiales*	1828019	1761	10881 8	36.1	86.58	0
Bin240	unclassified *Clostridiales*	2677664	2647	74195	39	81.54	0
Bin55	unclassified *Bacteroidales*	2708720	2177	29051	38.2	79.44	1.32
Bin150	*Lachnospiraceae*	3969489	3892	43630	41.3	91.14	4.75
Bin241	unclassified *Clostridiales*	1925525	1856	29444	37.2	84.34	2.01
Bin242	unclassified *Clostridiales*	2007844	1966	63047	39.9	99.19	2.15
Bin139	*Eubacteriaceae*	2181022	2255	49723	39	97.99	0.34
Bin243	unclassified *Clostridiales*	2180581	2277	64492	39.2	90.6	2.35

Bin244	unclassified *Clostridiales*	1706632	1538	111639	37.3	85.23	0
Bin245	unclassified *Clostridiales*	2489405	2376	333459	36.7	98.39	0
Bin246	unclassified *Clostridiales*	1973682	2003	15279	37	84.68	1.01
Bin151	*Lachnospiraceae*	4166508	4234	40172	39.4	94.74	3.01
Bin247	unclassified *Clostridiales*	3247545	2907	30463	38.4	96.64	4.7
Bin56	unclassified *Bacteroidales*	2740503	2362	17988	37.1	92.82	0.25
Bin248	unclassified *Clostridiales*	2205832	2206	47517	39.1	91.28	0.67
Bin249	unclassified *Clostridiales*	1931383	1908	115179	37.9	95.97	1.34
Bin57	unclassified *Bacteroidales*	2825891	2291	35700	37.2	95.47	0.13
Bin164	*Ruminococcaceae*	2700705	2571	61881	37.2	87.76	0
Bin250	unclassified *Clostridiales*	3131831	3090	89621	39.1	98.39	1.61
Bin353	*Succinatimonas*	2223481	2031	30915	39.2	87.65	2.39
Bin251	unclassified *Clostridiales*	1988121	1831	79148	38.3	92.6	1.34
Bin324	*Erysipelotrichaceae*	2205326	2115	40568	37.5	97.62	0.32
Bin252	unclassified *Clostridiales*	2481236	2266	124674	38.5	92.2	0
Bin20	*Coriobacteriaceae*	2710304	2436	200214	37.9	99.73	1.61
Bin253	unclassified *Clostridiales*	1592435	1443	79320	38.3	92.64	0.7
Bin136	*Clostridium bolteae*	3146713	3042	61365	41.3	97.47	0.63
Bin140	*Eubacterium*	2096058	1852	94653	39.8	96.45	0.95
Bin331	*Megasphaera*	2689152	2595	43150	38.2	94.31	0.6
Bin346	*Sutterella wadsworthensis*	2492678	2095	45587	33.9	97.52	1.24
Bin141	*Eubacterium*	2513023	2362	43094	39.8	94.68	0.63
Bin58	unclassified *Bacteroidales*	2086887	1704	35843	38.5	97.88	0.86
Bin330	*Phascolarctobacterium*	2170661	2130	13371	39.5	95.3	1.8
Bin254	unclassified *Clostridiales*	2482995	2189	25177	40.1	80.76	1.79
Bin255	unclassified *Clostridiales*	1970220	1878	49057	37.6	95.43	1.34

Bin256	unclassified *Clostridiales*	3029120	2799	36454	36.9	95.97	2.69
Bin191	*Ruminococcus*	2119190	2089	42295	37.7	95.99	1.41
Bin165	*Ruminococcaceae*	2451609	2347	32137	37.3	80.59	4.93
Bin142	*Eubacterium*	1699470	1770	6671	38.5	76.9	0
Bin257	unclassified *Clostridiales*	1739302	1714	6327	36.4	73.64	2.24
Bin129	*Lactobacillus johnsonii*	1907384	1930	28754	35.5	99.03	1.41
Bin258	unclassified *Clostridiales*	1888926	1812	44278	37.6	84.23	1.44
Bin347	*Dechloromonas aromatica*	1807772	1626	30276	34.8	89.57	0.47
Bin21	*Coriobacteriaceae*	1690747	1581	12055	36.2	94.83	3.13
Bin59	unclassified *Bacteroidales*	1681287	1479	11796	38	77.59	4.62
Bin259	unclassified *Clostridiales*	2936626	2740	23635	37.6	97.14	3.23
Bin260	unclassified *Clostridiales*	1293082	1395	8699	37.1	70.8	3.36
Bin204	*Subdoligranulum variabile*	2645506	2520	49156	36.5	80.25	2.04
Bin30	*Bacteroides*	5079918	4404	122658	39.2	88.84	3.41
Bin128	*Lactobacillus delbrueckii*	1477589	1451	8687	35.1	82.99	0.32
Bin340	*Veillonella*	2396989	2376	61398	38.2	99.4	2.45
Bin60	unclassified *Bacteroidales*	2910260	2518	16209	37.2	94.53	4.8
Bin261	unclassified *Clostridiales*	1266743	1367	5986	38.4	74.57	0.02
Bin354	*Succinatimonas*	1787287	1683	32177	37.5	88.79	4.18
Bin61	unclassified *Bacteroidales*	2457371	1802	18440	36.4	89.68	2.1
Bin166	*Ruminococcaceae*	2151898	2015	50019	38.1	93.88	0
Bin22	*Coriobacteriaceae*	1982127	1764	25401	36.3	98.28	3.29
Bin198	*Subdoligranulum*	2826653	2643	112415	35.6	98.3	0.87
Bin262	unclassified *Clostridiales*	1839038	1786	45360	37.7	88.95	0
Bin23	*Coriobacteriaceae*	1680601	1642	10116	39.1	90.5	2.79
Bin14	*Mycoplasmatales*	1616339	1891	30006	38.1	89.81	3.93
Bin263	unclassified *Clostridiales*	2291401	2388	7670	37.8	91.38	4.34
Bin264	unclassified *Clostridiales*	1575513	1469	33174	36.8	72.58	0
Bin265	unclassified *Clostridiales*	2531601	2366	72378	36.5	96.37	1.08

Bin266	unclassified *Clostridiales*	1879263	1986	13156	40.1	95.69	3.63
Bin267	unclassified *Clostridiales*	1622984	1614	21069	39.9	86.29	0
Bin268	unclassified *Clostridiales*	1668797	1733	8345	37.4	87.04	2.02
Bin167	*Ruminococcaceae*	2180112	2079	79115	36.1	97.99	0
Bin269	unclassified *Clostridiales*	1720739	1684	59249	39.5	90.03	0.81
Bin62	unclassified *Bacteroidales*	2977236	2328	34534	36.4	89.44	0.93
Bin9	*Treponema*	2110065	1920	22791	37.8	89.34	2.45
Bin63	unclassified *Bacteroidales*	2446683	1867	10328	37.7	75.72	2.22
Bin64	unclassified *Bacteroidales*	2267061	1753	52800	35.8	96.89	0
Bin344	*Pirellulaceae*	2332346	1705	48194	36.1	71.2	0
Bin270	unclassified *Clostridiales*	1946657	1999	33781	38.3	90.25	1.13
Bin271	unclassified *Clostridiales*	2061864	2158	65481	38.5	91.95	1.74
Bin65	unclassified *Bacteroidales*	2504743	1801	23786	37.6	73.58	0
Bin66	unclassified *Bacteroidales*	1989460	1749	26287	40.8	89.6	0.95
Bin67	unclassified *Bacteroidales*	1714397	1519	12313	40.8	81.7	4.11
Bin132	*Lactobacillus reuteri*	1496783	1473	10171	33.6	95.36	0
Bin272	unclassified *Clostridiales*	2656576	2708	66528	38.1	97.97	0
Bin342	*Lentisphaeraceae*	2078870	1625	55157	33.6	92.57	2.73
Bin187	*Faecalibacterium*	2246804	2143	35889	36.9	99.15	0.68
Bin68	unclassified *Bacteroidales*	2434754	2036	25781	36.5	96.56	2.7
Bin273	unclassified *Clostridiales*	2389425	2162	50319	38.2	74.73	3.9
Bin69	unclassified *Bacteroidales*	2322208	1840	29783	36.8	89.78	0.65
Bin274	unclassified *Clostridiales*	2261105	2150	70628	38.6	94.97	2.35
Bin130	*Lactobacillus johnsonii*	1293336	1334	42956	35.8	76.27	0
Bin10	*Treponema*	2569688	2332	81465	37.7	97.2	1.4
Bin275	unclassified *Clostridiales*	2802272	2803	66055	38	98.32	0.67
Bin70	unclassified *Bacteroidales*	2901996	2311	13684	37.3	79.43	2.75
Bin168	*Ruminococcaceae*	2047572	1877	87297	38.2	89.8	0.68
Bin328	*Phascolarctobacterium*	1465414	1561	7798	37.9	86.03	2.81
Bin143	*Eubacterium*	1881236	1888	17047	39.5	95.03	2.32

Bin276	unclassified *Clostridiales*	1969293	1902	102048	36.3	92.37	1.34
Bin71	unclassified *Bacteroidales*	2331089	1906	27233	35.9	94.76	1.09
Bin24	*Coriobacteriaceae*	2029779	1781	77820	36.4	99.19	3.51
Bin144	*Eubacterium*	1794056	1645	29179	40.8	95.77	3.55
Bin169	*Ruminococcaceae*	1697960	1738	5956	38.8	86.24	1.68
Bin205	unclassified *Clostridiales*	2131993	2073	185650	38.4	97.99	2.28
Bin277	unclassified *Clostridiales*	1897894	1922	14722	39.2	81.54	4.7
Bin192	*Ruminococcus*	2439570	2452	29950	37.3	97.99	4.87
Bin278	unclassified *Clostridiales*	2338762	2346	11343	36.8	86.35	4.81
Bin72	unclassified *Bacteroidales*	1514134	1343	15342	40.1	88.6	0.4
Bin73	unclassified *Bacteroidales*	2682180	2060	27344	38.6	90.64	4.78
Bin279	unclassified *Clostridiales*	2263384	2034	24333	39.1	95.11	3.36
Bin133	*Lactobacillus reuteri*	1886628	1840	13296	33.1	95.15	2.08
Bin5	*Escherichia coli*	4590617	4441	23061	36	97.05	1.21
Bin280	unclassified *Clostridiales*	1658702	1628	13452	40	92.34	0.34
Bin170	*Ruminococcaceae*	1700865	1782	5958	39.4	85.57	1.68
Bin332	*Megasphaera*	2278117	2230	71445	37.8	94.41	0
Bin199	*Subdoligranulum*	2928041	2799	161615	35.8	98.64	0
Bin336	*Mitsuokella*	2228453	2064	66232	34.8	86.45	0.08
Bin74	unclassified *Bacteroidales*	2484673	1793	20830	37.7	71.93	2.96
Bin75	unclassified *Bacteroidales*	2283003	1711	16966	37.1	80.96	1.23
Bin76	unclassified *Bacteroidales*	2650459	2007	25441	37.1	87.5	0.68
Bin281	unclassified *Clostridiales*	2055966	1969	25266	36.9	90.6	0.67
Bin282	unclassified *Clostridiales*	1371611	1433	9108	38.9	81.4	0
Bin283	unclassified *Clostridiales*	1198057	1313	33671	38.1	73.37	0.22
Bin15	*Mycoplasmatales*	1225896	1305	38939	40.7	94.38	3.37
Bin284	unclassified *Clostridiales*	2082480	2033	139880	36.9	90.77	0.7

Bin285	unclassified *Clostridiales*	1350016	1440	74085	38.2	74.21	0.89
Bin286	unclassified *Clostridiales*	1633266	1724	8598	40.4	94.19	2.42
Bin171	*Ruminococcaceae*	1766373	1743	11378	39.7	88.93	2.68
Bin77	unclassified *Bacteroidales*	2209579	2033	16917	38	95.86	3.39
Bin172	*Ruminococcaceae*	2032609	1797	28782	38.3	79.88	0.67
Bin287	unclassified *Clostridiales*	2719853	2651	119036	40.2	94.97	1.18
Bin78	unclassified *Bacteroidales*	2571420	2063	31496	37.9	89.56	3.46
Bin1	*Methanobrevibacter ruminantium*	2829865	3006	74937	40.4	99.2	4
Bin288	unclassified *Clostridiales*	1596527	1530	38000	40	91.16	1.57
Bin289	unclassified *Clostridiales*	2032812	1954	54383	37.6	84.45	4.7
Bin290	unclassified *Clostridiales*	2408428	2367	39986	36.4	96.37	1.08
Bin291	unclassified *Clostridiales*	2301193	2202	44239	38.8	92.06	4.99
Bin292	unclassified *Clostridiales*	1508701	1520	46168	39.6	79.48	0
Bin79	unclassified *Bacteroidales*	1728802	1594	6211	40.7	75.19	4.55
Bin80	unclassified *Bacteroidales*	2228938	1860	30674	35.6	89.31	0.88
Bin293	unclassified *Clostridiales*	2220870	2170	127927	38.3	97.99	0.84
Bin173	*Ruminococcaceae*	2753304	2574	20210	36.3	97.24	2.49
Bin174	*Ruminococcaceae*	2061437	1890	77005	39.5	97.05	0
Bin81	unclassified *Bacteroidales*	2644581	2267	11473	37.4	79.5	1.67
Bin82	unclassified *Bacteroidales*	2235881	1746	28470	35.9	94.61	1.49
Bin83	unclassified *Bacteroidales*	2173770	2099	9379	41.1	84.52	4.04
Bin145	*Eubacterium*	2021707	1983	15731	39	92.17	0.22
Bin25	*Coriobacteriaceae*	1217896	1272	5679	38.3	74.84	2.42
Bin84	unclassified *Bacteroidales*	2661534	2182	34087	36.5	91.12	3.21
Bin85	unclassified *Bacteroidales*	2290494	1976	11344	37.2	80.6	1.07
Bin86	unclassified *Bacteroidales*	2515548	2157	55863	35	86.54	0.69
Bin175	*Ruminococcaceae*	1372327	1374	42269	38.6	73.94	0.34
Bin87	unclassified *Bacteroidales*	2639495	2368	15850	38.6	88.29	0.19

Bin176	*Ruminococcaceae*	2518734	2617	24385	39.7	91.87	1.48
Bin88	unclassified *Bacteroidales*	1540644	1411	24417	40.4	75.37	0.48
Bin89	unclassified *Bacteroidales*	1656522	1426	15993	39.1	71.6	0
Bin90	unclassified *Bacteroidales*	2130712	1764	17352	36.3	74.18	1.67
Bin200	*Subdoligranulum*	2001516	1874	15682	35.6	92.78	2.61
Bin294	unclassified *Clostridiales*	1755699	1699	19784	39.9	90.53	1.36
Bin91	unclassified *Bacteroidales*	2996947	3051	27733	38.7	81.57	3.67
Bin92	unclassified *Bacteroidales*	2210670	2088	6682	37.3	83.34	1.13
Bin93	unclassified *Bacteroidales*	3309932	2960	19987	37.6	94.85	2.1
Bin94	unclassified *Bacteroidales*	1998319	1785	5581	36.4	73.15	2.94
Bin95	unclassified *Bacteroidales*	2896936	2689	34827	39.1	94.49	4.15
Bin295	unclassified *Clostridiales*	1747512	1751	93699	40.4	90.94	0.84
Bin96	unclassified *Bacteroidales*	1972060	1660	54107	41.5	85.56	1.54
Bin337	*Mitsuokella*	2227532	2060	20606	39.2	96.54	2.53
Bin360	*Unclassidied TM7*	2269311	1996	14377	37.4	74.36	3.08
Bin97	unclassified *Bacteroidales*	1824678	1592	12148	38.2	91.78	3.92
Bin296	unclassified *Clostridiales*	1734705	1714	41258	37.1	90.27	0.5
Bin125	*Lactobacillus amylovorus*	2092575	2282	17389	35.4	96.56	2.19
Bin134	*Lactobacillus reuteri*	1837333	1898	21850	34.4	95.99	0.55
Bin98	unclassified *Bacteroidales*	1854803	1591	17914	40.6	80.19	0.24
Bin195	*Ruminococcus flavefaciens*	2924649	2704	29607	38.7	90.94	0.06
Bin351	*Myxococcales*	3174537	2385	20315	36.7	76.65	1.77
Bin297	unclassified *Clostridiales*	1894814	1864	56400	38.8	90.81	0.34
Bin298	unclassified *Clostridiales*	1754008	1780	22966	36.1	82.66	0.22
Bin131	*Lactobacillus johnsonii*	2354155	2481	22259	36.1	97.77	3.54
Bin177	*Ruminococcaceae*	2189092	2103	47065	39.3	97.27	0
Bin99	unclassified *Bacteroidales*	2248797	1866	34014	40.6	93.65	0.21
Bin329	*Phascolarctobacterium*	1335061	1402	6254	37.9	74.09	2.84
Bin100	unclassified *Bacteroidales*	1996743	1652	7938	35.4	76.95	3.04
Bin178	*Ruminococcaceae*	2132237	2012	59826	36.3	97.99	0
Bin101	unclassified *Bacteroidales*	2086320	1717	19705	36.1	70.02	1.85

Bin152	*Lachnospiraceae*	2294616	2307	16503	39	87.87	4.35
Bin352	*Succinivibrionaceae*	2691173	2290	21390	39.9	94.49	2.11
Bin299	unclassified *Clostridiales*	2379421	2445	11275	36.9	90.25	3.02
Bin102	unclassified *Bacteroidales*	2131512	1807	16617	35.7	84.68	4.41
Bin148	*Lachnospiraceae*	2241106	1906	73454	36.5	96.38	0
Bin103	unclassified *Bacteroidales*	1875526	1663	5248	36.7	77.5	2.67
Bin26	*Coriobacteriaceae*	2012174	1990	8524	36.8	87.12	3.84
Bin300	unclassified *Clostridiales*	1446482	1501	8285	37	70.38	1.34
Bin135	*Clostridiaceae*	2321535	2307	15848	39.7	91.85	4.43
Bin27	*Coriobacteriaceae*	1859870	1738	20475	37.5	94.76	0.44
Bin301	unclassified *Clostridiales*	2620908	2503	8128	35.9	86.75	3.85
Bin302	unclassified *Clostridiales*	1762539	1742	46932	40.1	90.92	2.68
Bin104	unclassified *Bacteroidales*	2043352	1734	17974	39.5	81.95	1.35
Bin105	unclassified *Bacteroidales*	2115922	1845	18073	34.8	86.51	1.28
Bin106	unclassified *Bacteroidales*	3360894	2818	45863	39.6	91.95	1.3
Bin179	*Ruminococcaceae*	1733732	1623	35547	38.6	87.7	0
Bin303	unclassified *Clostridiales*	1522075	1530	9711	36.9	71.1	3.91
Bin304	unclassified *Clostridiales*	2277679	2081	68008	37.8	96.61	0
Bin6	*Escherichia coli*	4378339	4166	56008	36.2	98.1	0.51
Bin305	unclassified *Clostridiales*	1556708	1484	22206	39.6	87.88	0.81
Bin107	unclassified *Bacteroidales*	2422660	1843	17841	37.2	86.9	2.04
Bin338	*Mitsuokella*	2584130	2609	29763	38	83.51	1.83
Bin108	unclassified *Bacteroidales*	2212806	1742	8802	36.5	84.39	2.92
Bin306	unclassified *Clostridiales*	1931836	1760	14049	36.8	91.89	1.8
Bin153	*Lachnospiraceae*	2396817	2496	19514	38.9	91.24	2.86
Bin307	unclassified *Clostridiales*	2977250	2889	38767	38.5	97.55	4.36
Bin109	unclassified *Bacteroidales*	2039448	1844	20369	40.8	82.77	1.67
Bin126	*Lactobacillus amylovorus*	1872704	2024	21308	35.9	93.1	3.63
Bin11	*Treponema*	3713748	3443	23790	37.8	92.83	4.46
Bin180	*Ruminococcaceae*	1184430	1215	14014	38.3	72.62	3.8
Bin181	*Ruminococcaceae*	1370630	1410	6479	39.7	73.17	1.51

Bin127	*Lactobacillus amylovorus*	2305390	2673	7851	35.5	71.87	4.85
Bin308	unclassified *Clostridiales*	2776932	2584	9135	37.7	80.16	3.76
Bin309	unclassified *Clostridiales*	1485439	1472	53265	39.1	71.14	0.67
Bin110	unclassified *Bacteroidales*	2318070	1921	34010	34.4	93.21	0.75
Bin339	*Mitsuokella multacida*	2280648	2057	73000	36.8	99.07	0.23
Bin16	*Mycoplasmatales*	1360808	1457	85127	39.7	89.16	2.81
Bin111	unclassified *Bacteroidales*	1831141	1544	14930	36.7	90.49	0.81
Bin112	unclassified *Bacteroidales*	2036153	1766	18811	37.9	74.03	1.79
Bin310	unclassified *Clostridiales*	1569787	1638	15266	37.2	88.96	4.88
Bin311	unclassified *Clostridiales*	1403937	1349	9202	38.4	72.66	2.01
Bin188	*Faecalibacterium*	1692888	1597	25270	37	86.52	2.63
Bin350	*Desulfovibrio piger*	2137733	1868	21919	38.7	88.94	0.59
Bin7	*Escherichia coli*	3690123	3667	9802	36.1	81.73	0.75
Bin312	unclassified *Clostridiales*	1431094	1344	19208	36.5	70.94	4.36
Bin113	unclassified *Bacteroidales*	1797376	1739	5314	41.2	73.41	4.48
Bin333	*Megasphaera*	2626568	2595	52653	37.9	96.31	0.81
Bin313	unclassified *Clostridiales*	1985734	2068	45508	38.6	89.85	2.85
Bin189	*Faecalibacterium*	2148852	2039	32749	36.9	97.49	2.35
Bin314	unclassified *Clostridiales*	2648966	2871	19098	37.3	96.31	0.67
Bin201	*Subdoligranulum*	1964712	1779	58535	35.7	95.15	0
Bin114	unclassified *Bacteroidales*	1783367	1600	10225	40.6	77.19	2.14
Bin182	*Ruminococcaceae*	1826106	1887	13823	39.4	90.02	2.68
Bin315	unclassified *Clostridiales*	1658327	1638	17084	37.3	80.33	4.92
Bin146	*Eubacterium*	2527670	2706	6884	38.7	83.24	3.24
Bin115	unclassified *Bacteroidales*	2210555	1714	22129	37.1	75.05	4.26
Bin316	unclassified *Clostridiales*	1446827	1562	7106	40.1	88.72	0.81
Bin317	unclassified *Clostridiales*	1811894	1782	6272	36.9	87.38	2.24
Bin116	unclassified *Bacteroidales*	2337932	2206	19912	36.3	92.21	4.3
Bin117	unclassified *Bacteroidales*	2190512	2047	18531	41.5	82.96	3.31
Bin118	unclassified *Bacteroidales*	1923735	1995	7304	40.7	79	4.31
Bin183	*Ruminococcaceae*	1406707	1453	21106	38.5	74.61	2.01

Bin334	*Megasphaera*	2241385	2084	49478	38	98.3	0.06
Bin184	*Ruminococcaceae*	2048608	1914	79805	36.6	91.95	0.67
Bin343	*Lentisphaeraceae*	2004189	1701	23419	37.5	86.55	1.35
Bin119	unclassified *Bacteroidales*	1964288	1745	14945	41.7	86.29	2.57
Bin318	unclassified *Clostridiales*	2153926	2062	61047	38.6	96.98	1.12
Bin319	unclassified *Clostridiales*	1343990	1432	6359	38.3	84.02	2.8
Bin320	unclassified *Clostridiales*	1796414	1906	14183	36.1	89.71	0.34
Bin120	unclassified *Bacteroidales*	2333386	1866	24877	37	87.22	3.43
Bin121	unclassified *Bacteroidales*	2067332	1911	31884	38.1	94.34	1.08
Bin190	*Faecalibacterium*	1941345	1912	26562	36.9	95.58	4.55
Bin185	*Ruminococcaceae*	2281111	2023	17389	36.2	96.71	0
Bin335	*Megasphaera*	2010916	1896	31707	38	96.77	0.12
Bin321	unclassified *Clostridiales*	2124251	2120	12928	36.9	87.76	4.42
Bin322	unclassified *Clostridiales*	1645263	1730	7880	39.2	76.17	1.98
Bin28	*Coriobacteriaceae*	1696648	1711	6161	36.5	72.53	4.49
Bin325	*Erysipelotrichaceae*	2212377	2170	35697	38	89.52	3.17
Bin17	*Mycoplasmatales*	1819014	2104	47690	40	91.29	3.68
Bin323	unclassified *Clostridiales*	1311736	1539	6551	37.4	73.97	0.67
Bin122	unclassified *Bacteroidales*	2259638	1814	20780	36.1	95.46	1.35
Bin202	*Subdoligranulum*	1999819	1919	10853	35.4	86.37	2.04
Bin123	unclassified *Bacteroidales*	2100023	1942	8678	40.5	77.07	3.61
Bin124	unclassified *Bacteroidales*	1873840	1589	6907	36.3	70.96	4.28
Bin361		1310798	1491	6067	36.3	69.22	2.46
Bin362		2973976	3198	11671	37.9	69.08	9.92
Bin363		2468156	1917	14979	37.6	68.92	0.37
Bin364		2233528	2166	8986	38.9	68.9	5.64
				13548			
Bin365		1603743	1515	8	37.3	68.6	0
Bin366		1129220	1317	8837	39.5	68.46	2.25
Bin367		2562344	2478	29474	38.2	68.28	2.15
Bin368		1854379	1923	4949	39.1	68.23	5.14

Bin369	2167306	1648	20958	40.5	68.1	0
Bin370	1694210	1815	13649	40.3	67.94	8.95
Bin371	1503394	1597	4467	38.5	67.93	9.55
Bin372	2002013	1867	5452	35.2	67.82	4.14
Bin373	2021820	1856	6342	39.3	67.72	6.54
Bin374	1791261	1951	5299	38.8	67.65	5.48
Bin375	1365044	1532	4677	40.2	67.52	7.27
Bin376	1934432	1818	5647	36.9	67.5	2.83
Bin377	1153419	1198	4548	39.4	67.48	1.96
Bin378	1334982	1358	6963	38.5	67.4	3.02
Bin379	2408349	2434	6818	38.9	67.24	6.9
Bin380	1254731	1319	4818	39.7	67.21	2.24
Bin381	1102516	1163	5485	38.5	67.01	3.37
Bin382	1212776	1346	3911	39.7	66.78	5.43
Bin383	1856568	1620	10874	37.2	66.51	2.71
Bin384	1642157	1714	3888	40.7	66.45	6.38
Bin385	1535590	1609	6355	37.8	66.38	1.72
Bin386	2398121	1952	14140	36.6	66	3.83
Bin387	1506353	1587	11286	39.6	65.88	0
Bin388	2440555	2614	3811	39	65.86	6.88
Bin389	1659016	1442	36082	37	65.66	2.01
Bin390	1511610	1660	5072	36.8	65.07	5.12
Bin391	1965207	1772	39194	39.5	65	0.31
Bin392	1232678	1261	20585	39.3	64.89	1.61
Bin393	2139668	1804	10600	37.3	64.66	0
Bin394	2172764	1664	27278	35.3	64.66	0
Bin395	1056935	1081	4378	39	64.48	0
Bin396	1044913	1046	5361	39.4	64.43	4.15
Bin397	1120639	1149	5002	39	63.81	0.87
Bin398	1524997	1461	74311	40.1	63.76	1.51
Bin399	1315030	1456	4306	38	63.6	5.27

Bin400	1252940	1425	4289	40	63.53	8.9
Bin401	2030149	1934	28568	39.2	63.5	7.52
Bin402	2285915	2649	4969	38.2	63.44	7.27
			83645			
Bin403	1063973	1091	4	34.9	63.25	0.85
Bin404	1302128	1449	7144	34.7	63.22	8.83
Bin405	1193006	1216	5177	38.7	63.21	0.16
Bin406	1357436	1402	6822	36.7	63.11	5.09
Bin407	1107204	1206	3296	38.2	63.07	5.78
Bin408	1336841	1361	7767	39.5	63.05	2.42
Bin409	1306560	1345	8121	39.6	62.74	3.02
Bin410	1410508	1508	5069	38	62.6	0
Bin411	2039121	1502	37377	36	62.41	0.37
Bin412	1428703	1406	5019	36.5	62.36	2.95
Bin413	1857618	2022	8047	39.5	62.07	6.9
Bin414	3195805	3548	17061	37.2	62.03	8.98
Bin415	2077190	1553	9600	37.8	61.92	0.19
Bin416	1749183	1859	13581	40.1	61.89	3.02
Bin417	1459340	1610	6369	37.3	61.51	0.67
Bin418	1703018	1854	25477	37.3	61.3	8.35
Bin419	1267823	1405	3491	39.3	60.84	4.5
Bin420	2144216	2460	3585	38.5	60.8	5.24
Bin421	1519910	1514	69773	36.8	60.74	9.4
Bin422	1525945	1310	7972	37	60.65	2
Bin423	1967855	1657	4061	37.7	60.46	8.87
Bin424	1223863	1515	5767	40	60.29	7.72
Bin425	1656526	1734	7527	36.7	60.06	3.45
Bin426	2277586	2402	8574	40.5	60.05	3.55
Bin427	981984	1066	4128	38.4	60.05	0.37
Bin428	1288927	1406	4986	37.6	59.99	0.81
Bin429	1843394	1622	13816	36.8	59.84	0.38

Bin430	1130162	1237	7522	38.9	59.74	1.68
Bin431	1909114	1968	3645	40.7	59.71	8.65
Bin432	1272744	1229	7493	40.5	59.67	1.9
Bin433	1808196	1674	4909	39.2	59.38	6.09
Bin434	1123184	1154	5452	38.4	59.32	3.19
Bin435	1830656	1883	12955	36.8	58.93	6.25
Bin436	2043955	1660	16959	36.5	58.71	4.83
Bin437	2064895	1928	16798	39.1	58.62	0
Bin438	1304382	1359	8412	37	58.62	0
Bin439	1122848	1199	4205	40.3	58.57	0.4
Bin440	1447039	1424	9281	34.8	58.18	1.13
Bin441	2223136	1955	17178	38.2	58.02	4.25
Bin442	1293223	1438	3945	37.8	57.96	5.11
Bin443	1090948	1229	3835	40.4	57.89	5.26
Bin444	1200336	1262	5428	35.8	57.78	1.34
Bin445	2722530	2478	7284	38.5	57.76	1.72
Bin446	893826	903	14221	38	57.76	6.11
Bin447	1909199	1522	6579	36.9	57.68	8.54
Bin448	1316469	1514	4241	40.8	57.47	5.17
Bin449	2035486	1811	12610	37.7	57.36	7.59
Bin450	2092590	1928	8112	37.5	57.24	5.17
Bin451	1682184	1750	6544	40.4	57.06	4.71
Bin452	2365437	2292	5550	38.9	56.99	2
Bin453	1157956	1262	3910	37.5	56.61	0.81
Bin454	1095620	1119	3432	35.9	56.54	2.96
Bin455	1627873	1919	5148	38.8	56.53	1.59
Bin456	1072942	1190	4897	39.7	56.33	1.43
Bin457	744099	764	92760	34.8	55.74	3.7
Bin458	999476	1026	16143	40	55.65	0.7
Bin459	824290	917	27926	34.8	55.56	1.28
Bin460	961899	1030	11126	38.4	55.52	0

Bin461	1744531	1932	5603	36.5	55.45	8
Bin462	1577161	1363	4639	38.9	55.29	3.62
Bin463	1471716	1336	32785	39.6	54.83	2.01
Bin464	1044685	1110	5323	38.9	54.66	1.72
Bin465	1430451	1550	3388	39.9	54.58	9.11
Bin466	1664482	1854	4840	38.9	54.39	3.51
Bin467	2396819	2601	6838	36.9	54.37	9.28
Bin468	956092	997	3877	38.7	54.21	0.48
Bin469	1151845	1018	18326	36	54.16	0
Bin470	1353123	1450	6502	40.2	54.02	5.17
Bin471	1365045	1425	4217	36.6	54	3.77
Bin472	842914	883	6381	40.2	53.99	2.24
Bin473	1592989	1657	3434	40.6	53.87	5.34
Bin474	1464852	1649	6949	39.8	53.79	6.9
Bin475	1166255	1352	4345	39	53.68	7.48
Bin476	1110080	1169	3864	38.5	53.64	4.64
Bin477	1015727	1000	11865	37.7	53.61	9.48
Bin478	1364517	1394	40544	37	53.5	1.68
Bin479	2036698	1468	16371	37.6	53.45	0
Bin480	1075416	1293	4199	38.1	53.19	2.06
Bin481	1145424	1310	3767	37.9	52.9	5.17
Bin482	1408638	1398	5317	36.9	52.71	5.26
Bin483	953388	955	70335	40.6	52.66	0
Bin484	1606183	1547	3686	35.4	52.58	4.26
Bin485	1845347	1502	6534	37.7	52.35	0
Bin486	1839645	1864	4762	37.7	51.72	3.45
Bin487	999395	1130	4498	39.3	51.72	1.72
Bin488	1854460	1688	7261	39.2	51.65	0.86
Bin489	1476623	1456	3224	36.6	51.23	6.02
Bin490	2003427	2397	3148	38.7	51.22	7.86
Bin491	1686034	1759	4743	39	51.03	1.51

Bin492	1108364	1316	3442	39.1	51.01	3.6
Bin493	1017259	1054	12793	38.4	51.01	3.69
Bin494	1139929	1254	13351	37.3	50.99	4.7
Bin495	2194692	2423	3257	38.9	50.92	8.39
Bin496	1173324	1218	5158	38.2	50.17	1.72
Bin497	1866523	2090	3858	35.8	50.16	7.14
Bin498	1126168	1359	3610	38.5	50.03	0.84
Bin499	3277796	2985	32767	38	99.14	5.02
Bin500	1966701	1981	9973	38.3	85.7	5.02
Bin501	2240957	2115	12341	39.8	76.72	5.02
Bin502	1793018	1764	27158	39.2	77.56	5.15
Bin503	1976925	1801	7832	36.9	75.22	5.16
Bin504	1878262	1807	10672	36.3	98.28	5.17
Bin505	1426083	1421	8193	33.2	70.69	5.17
Bin506	2662936	2432	85913	38.8	92.34	5.2
Bin507	2139673	2220	9920	38.8	79.86	5.21
Bin508	1637023	1743	4711	37.3	79.79	5.24
Bin509	2372517	1857	16259	36.4	76.5	5.25
Bin510	2598569	2210	54716	36.6	94.83	5.33
Bin511	1824212	1881	12493	38.9	87.19	5.37
Bin512	2136769	1883	52688	35.6	88.64	5.49
Bin513	2459858	2223	61970	37.1	97.58	5.5
Bin514	1918557	1827	4882	37.1	70.86	5.53
Bin515	1983956	1854	11355	38.1	92.92	5.54
Bin516	2411490	2409	14271	37	91.44	5.59
Bin517	1152004	1307	9552	38.6	75.87	5.62
Bin518	2291540	1748	63204	37.2	71.22	5.66
Bin519	2573945	2477	99043	36.9	100	5.68
Bin520	2466033	2084	10316	36.6	85.85	5.72
Bin521	2241228	2136	25545	37.7	94.46	5.74
Bin522	1741172	1637	19547	40.3	87.7	5.76

Bin523	3589326	3446	54690	38.5	96.86	5.77
Bin524	1740354	1747	15838	36.8	75.62	5.81
Bin525	2571745	2634	10356	37.8	89.28	5.83
Bin526	2200911	2154	14936	37	94.95	5.88
Bin527	1966188	1917	7969	38.1	81.17	5.91
Bin528	1799007	1749	16035	36.8	73.23	5.91
Bin529	2332343	2481	14957	37.4	87.27	5.98
Bin530	1378029	1432	7085	39.5	78.13	5.99
Bin531	1600341	1603	4978	39	71.3	6.03
Bin532	4164407	4078	81930	38	97.99	6.04
Bin533	1343577	1506	6698	37	74.13	6.04
Bin534	1238049	1302	57401	38.4	92.13	6.18
Bin535	1717864	1820	9895	40.1	98.19	6.26
Bin536	1960524	1855	16968	35.4	92.03	6.46
Bin537	1751733	1780	13736	36.9	91.72	6.54
Bin538	4104711	4144	61491	38.4	98.55	6.57
Bin539	1878582	1836	30545	39.7	79.98	6.6
Bin540	2185120	2110	42883	38.6	95.34	6.61
Bin541	1929332	1942	6011	36.9	84.82	6.62
Bin542	1520479	1576	41939	40.2	84.83	6.63
Bin543	3287706	3151	16655	38.7	94.13	6.66
Bin544	3054476	3236	13676	35.6	90.73	6.72
Bin545	2717093	2490	9993	35.8	93.01	6.76
Bin546	1776760	1937	11635	38.9	80.98	6.83
Bin547	1942522	2176	9039	39	78.61	6.94
Bin548	1706268	1800	10885	38.2	79.25	6.96
Bin549	1764100	1855	4871	37	76.71	7.03
Bin550	1954045	1846	62478	39.5	90.23	7.05
Bin551	1545271	1691	5163	40	77.74	7.05
Bin552	1937801	1941	16548	36.6	91.61	7.13
Bin553	1955867	1803	7391	40.7	74.36	7.14

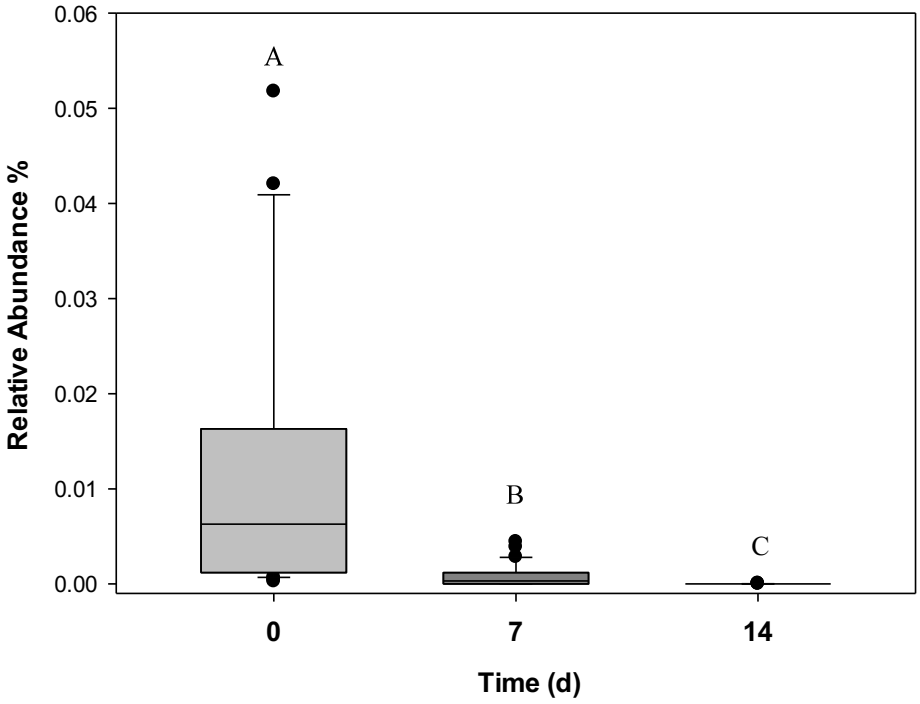
Bin554	2106644	1950	28768	36.9	94.74	7.18
Bin555	3258633	2713	63761	37.5	99.02	7.24
Bin556	2299186	2301	9415	40.4	83.44	7.27
Bin557	4074606	4164	27117	41.1	87.97	7.34
Bin558	1570448	1724	7931	40.3	98.28	7.37
Bin559	2155938	2049	7414	41	85.64	7.57
Bin560	1759147	1611	11959	40.6	84.43	7.59
Bin561	2177353	2360	5738	39.6	75.56	7.65
Bin562	1839667	1827	8586	38.1	87.15	7.66
Bin563	2490848	2247	22329	36.8	89.66	7.68
Bin564	3321712	2936	9454	37.4	89.72	7.69
Bin565	1362611	1281	16653	40.7	95.51	7.72
Bin566	2530947	2023	44585	34.3	93.1	7.84
Bin567	2550816	2698	19736	38.8	86.77	7.88
Bin568	1809625	1993	10322	39.3	82.62	7.9
Bin569	1702724	2116	4591	38.8	76.16	8.04
Bin570	2376943	2332	60127	39.3	97.32	8.22
Bin571	3694456	3203	49266	37.2	95.66	8.33
Bin572	2130877	2048	4929	40.1	73.07	8.35
Bin573	2198409	2063	45884	37	92.66	8.45
Bin574	2185721	1714	20828	35.5	89.76	8.49
Bin575	2341095	2108	10185	40.5	88.79	8.57
Bin576	3237146	3196	28485	39.1	80.76	8.63
Bin577	2816250	2525	11911	36.8	92.83	8.65
Bin578	1715919	1822	7148	36.8	71.46	8.67
Bin579	3354197	3573	19203	40.3	93.57	8.89
Bin580	1952089	1881	4954	40.5	73.81	8.93
Bin581	2952581	2721	21077	38.5	94.75	8.96
Bin582	2439996	2562	15432	37.7	91.72	9.06
Bin583	2534311	2460	59550	38.8	94.97	9.08
Bin584	2544605	2377	17895	39.7	88.62	9.16

Bin585	2739071	2851	30885	39.6	99.11	9.37
Bin586	2386437	2244	8616	40.6	86.97	9.38
Bin587	2051823	1759	7224	35.2	73.09	9.38
Bin588	3682531	4100	15862	37.9	89.07	9.4
Bin589	1826271	1831	17189	37.9	79.31	9.4
Bin590	2153349	2084	52811	37.9	83.05	9.53
Bin591	2624229	2313	21667	36	94.88	9.59
Bin592	2189278	1994	12534	36.8	92.66	9.63
Bin593	2040605	1931	8579	35.9	92.21	9.68
Bin594	2077685	2073	32982	37.9	92.89	9.73
Bin595	4004257	3782	43413	38.6	96.19	9.92
Bin596	2310127	2396	13485	37.5	91.11	9.98

Table S6.4. Accession number and reference to the biochemical characterization of enzymes BLAST for starch, fructan and lactose degradation

Name	Protein	UniProt accession #	Reference
Amy1_Bat	Beta/alpha-amylase	A0A2N0UIC8	[355]
Amy4_Bat	Alpha-amylase	A0A2N0UX89	[355]
Amy12_Fp	Pullulanase	A0A2N0UU23	[355]
GlgB_Fic	1,4-alpha-glucan branching enzyme GlgB	P30539	[342]
susG_Bt	Neopullulanase SusG	Q8A1G3	[356]
susA_Bat	Neopullulanase SusA	Q8A1G0	[356]
susB_Bat	Glucan 1,4-alpha-glucosidase SusB	G8JZS4	[356]
BT_3082_Bat	2,6-beta-D-fructofuranosidase	Q8A373	[321]
BT_1765_Bat	Levanase (2,6-beta-D-fructofuranosidase)	Q8A6W1	[321]
BT_1760_Bat	Glycoside hydrolase family 32	Q8A6W6	[321]
BT_1754_Bat	Two-component system sensor histidine kinase/response regulator	Q8A6X1	[321]
inuJ_Lb	Inulosucrase	Q74K42	[357]
sacA_Fic	Sucrose-6-phosphate hydrolase	A0A173R035	[316]
scrB_Lb	β-fructofuranosidase	D0R647	[358]
LacA_Lb	Beta-galactosidase LacA	C6H178	[359]
BbgI_Fic	Beta-galactosidase BbgII	D4QFE6	[360]
BbgIII_Fic	Beta-galactosidase BbgIII	A4K5H9	[360]
BbgIV_Fic	Beta-galactosidase BbgIV	D4QFE8	[360]
LacM_Lb	Beta-galactosidase LacM	Q02604	[361]

Figure S6.2. Relative abundance (%) of *Lactobacillus delbrueckii* of suckling pigs (day 0) and weaned pigs (day 7 and day 14) determined by Illumina sequencing of 16S tags. Data with unlike letters are significantly different ($P < 0.05$).



Chapter 7 Biochemical characterization of *glgB* gene cloned from swine intestinal bacteria

7.1. Introduction

Glucan-branching enzyme (EC:2.4.1.18) catalyzes the cleavage of α-(1→4)-glucan chain and transfer to generate α-(1→6)- glycosidic bonds which is common found in nature [362,363]. Among prokaryotes, two distinct types of glucan-branching enzyme (GH13 and GH57) [364,365] are identified with extremely diverse distribution within and between different phyla [364]. The spectrum of branching enzymes in gut microbiome also differentiates between hosts, among which pigs and human share the most similarity in branching enzyme profile [366].

In a previous study on metagenomic reconstructions of swine gut microbial metabolism [267] (Chapter 4), *Firmicutes* and *Bacteriodetes* harbored distinct starch-utilization system. *Firmicutes* dominated in extracellular glycosidases while *Bacteriodetes* possessed all the periplasmic amylolytic enzymes. This uneven distribution of glycosidases in the two dominant phyla of swine gut microbiota suggested a high level of metabolic cooperativity in starch utilization between these microorganisms. Glucan-branching enzyme *glgB* was the most abundant extracellular glycosidases found in *Firmicutes*, which highlighted its importance in primary degradation of starch in swine intestine. This enzyme converts unbranched substrates into highly branched structures and increased their accessibility to glycosidases and solubility [367]. However, these findings were concluded from metagenomic-assembled genomic sequence analysis, the precise elucidation of its role in starch utilization still requires biochemical validation.

In addition, the capacity of producing α-(1→6)- glucan also makes *glgB* highly useful in developing functional food and improving food quality. One example is adding α-(1→6)-linkages to maltodextrin to produce highly-branched dietary fiber, which is more resistant to intestinal

digestion and slowed to release glucose [368,369]. Addition of branching enzyme in wheat bread resulted in an increased bread volume and decreased crumb firmness [370]. Branching enzyme treatment also could retard retrogradation of corn starch, corresponding to a slowed hardness increase and taste declination of corn-starch-based food during storage [371].

The aim of this study is to verify the predicted enzymatic activity of *glgB* in formation of α-(1→6)-bonds. Two *glgB* gene variants encoding α-(1→4)-glucan branching enzyme were transferred from swine intestinal bacteria to *E. coli*. Confirming enzymatic activity will improve the elucidation of metabolic cooperativity between *Firmicutes* and *Bacteriodetes* in starch utilization in swine intestine and promote potential applications of *glgB* in food industry.

7.2. Materials and methods

7.2.1. Bacteria, plasmid and growth condition

E. coli BL21 Star (DE3) (Invitrogen) with pET-28b+ (Novagen, Etobicoke, ON, Canada) was cultivated in Luria-Bertani (LB) broth containing 0.05 g/L kanamycin and aerobically incubated at 37 ºC.

7.2.2. Prediction and validation of *glgB* gene sequences from swine intestinal bacteria

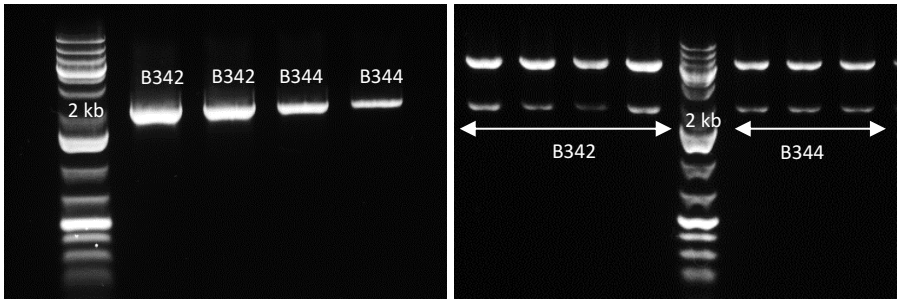
Two protein sequences with an amino acid identity of ≥ 40% against α - (1→4)-glucan branching-enzyme glgB (EC:2.4.1.18) of *Butyrivibrio fibrisolvens* [342] were selected from metagenomic-assembled proteomes of swine intestinal bacteria. Corresponding nucleotides sequences are listed in table S7.1. To validate these sequences, specific amplification of B342 and B344 was performed with high-fidelity Tag DNA polymerase (Platinum™ *Taq* DNA Polymerase High Fidelity, Invitrogen) from swine-feces isolated DNA mixture. SacI / SalI restriction enzyme cutting sites were also inserted for further analysis by PCR amplification with primers as following:

B34F-SacI (5’-3’): GCTGAGCTCATGACAACTGTAGAAAAGAAA;

B34R-SalI (5’-3’): GAAGTCGACGAATTCAAATACCGCAACG.

PCR products were cleaned and then preliminary checked by 1% agarose gel (1941 bps, Figure 7.1, left).

Figure 7.1. Validation of PCR amplification and insertion of *glgB* gene clone B342 and B344

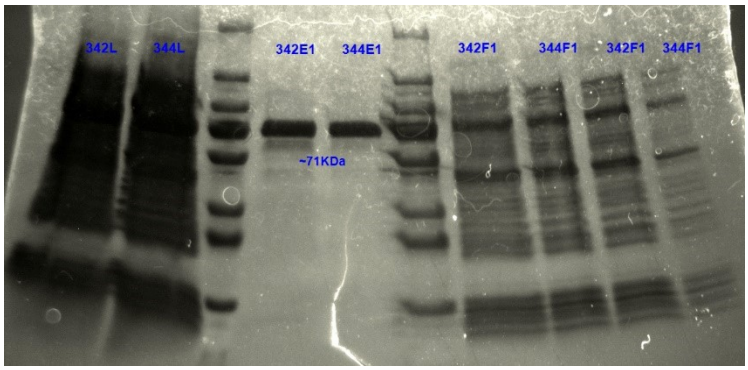


7.2.3. Branching enzyme *glgB* gene cloning and protein purification

Purified PCR amplicons and pET-28b+ vector was digested by SacI / SalI restriction enzymes and then ligated into recombinant plasmids following SacI / SalI digestion protocol (Figure 7.2, right). The recombinant plasmids were transferred into *E. coli* BL21 Star (DE3) chemically competent cells (Invitrogen, USA) for protein expression under the guidelines of One Shot BL21 (DE3) Competent cell Manual (Invitrogen, MAN0000662). Overexpression of *glgB* in *E. coli* was induced by 0.1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG).

After overnight growth, recombinant plasmid DNAs were isolated and then sequenced with T7 primer 5'- TAATACGACTCACTATAGGG -3' (University of Alberta, Faculty of Science, Canada). GlgB proteins were purified from colonies cloned with correct *glgB* gene using HisPur™ Ni-NTA Resin (Fisher Scientific, Loughborough, England) and validated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 7.2, right). Protein concentration was measured by Bio-Rad protein assay dye reagent (Bio-Rad, Mississauga, Canada) by using bovine serum albumin as the standard.

Figure 7.2. SDS-PAGE analysis of BL21 Star™ (DE3) expressed protein encoded by B342 and B344. Lanes: 1-2, cell extract of B342 and B344; 2, ladder; 3-4, the 1st elution of B342 and B344; 5-6, the 1st flow through of B342 and B344; 7-8, the 2nd flow through of B342 and B344.



7.2.4. Biochemical characterization of branching enzyme *glgB* gene clone

Branching activity of purified *glgB* from clone B342 and B344 against α - (1→4)-glucan were determined by the reduction of iodine-binding amylose in raw / heated (85 °C, 10 min) potato amylose (purity > 98%, Megazyme, Ireland) after 4h digestion (pH 7.4) as previously described [342]. A standard curve was generated from various concentration of heated amylose ranging from 0 to 100 mg /L. The quantitative detection of iodine-binding amylose was conducted based on the liner correlation between iodine-binding amylose content and the corresponding absorption of amylose-iodine complex at λ_{620nm}.

7.2.5. Statistical analysis

Differences of *glgB* branching enzyme activity on raw / heated amylose between *E. coli* with or without *glgB* cloned, or variations between eluted proteins and washing flow-through were compared by one-way ANOVA in R (version 3.4.3). Results are presented as means ± standard deviation. Comparisons with *P* values < 0.05 were considered significant different.

7.3. Results

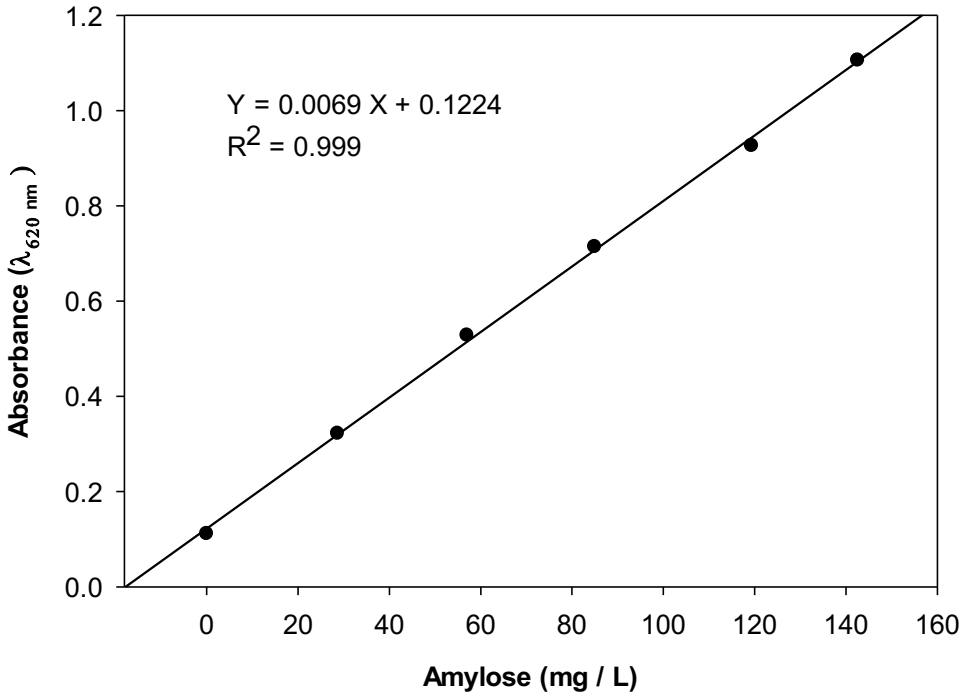
7.3.1. Cloning *glgB* gene from swine intestinal bacteria in *E. coli*

Initially, the existence of *glgB* gene were predicted based on annotation of metagenomic-assembled genomes of swine intestinal *Firmicutes* bacteria. Although it was still hard to identify which microorganisms harbored this enzyme, their sequences were verified by specific PCR amplification and sequencing (TableS7.1, Figure7.1). To validate the prediction of branching activity against α- (1→4)-glucan, *glgB* was cloned in *E. coli* and induced over expression. Clone B342 and B344 expressed branching-enzyme *glgB*, which was successfully purified and verified by SDS-PAGE (Figure7.2)

7.3.2. Quantitative determination of amylose by measuring absorption of amylose-iodine-complex

To establish quantitative detection of iodine-binding amylose, a standard curve was generated from various amylose amounts reflecting the linear relationship between iodine-binding amylose content and absorption of amylose-iodine-complex (Figure7.3). Raw potato amylose is partially soluble which requires heat treatment at 80 °C in water for 10 min to obtain uniform gelatinized solution [372]. Iodine-binding amylose detected the partial solubility of raw potato amylose approximately accounted for 34% of the total mass in this study (data no show) , which could serve as the initial content of iodine-binding amylose in raw potato amylose [372].

Figure 7.3. Standard curve for branching enzyme activity determination based on the decrease in absorption of amylose-iodine complex



7.3.3. Verification of retained enzymatic activity of purified *glgB*

Reductions of iodine-binding amylose between adding protein elution and washing flow-through was compared to verify that the retained enzyme activity of *glgB* after Ni-NTA spin column-based purification process. Compared to no protein negative control, purified protein significantly reduced the content of iodine-binding amylose detected in potato amylose while no significant difference detected in flow-through treated group after 4 h incubation ($p < 0.05$, data no shown). Meanwhile, adding cell lysate also significantly decreased the level of iodine-binding amylose in potato amylose ($p < 0.05$). No significant difference was observed between cell lysate and purified protein confirmed the retained enzyme activity of *glgB* (Table7.3, Figure7.4).

Table 7.1. Concentration of iodine-binding amylose in raw or heated potato amylose after digestion of clone B342 and B344 expressed protein. Data in the same column with different letter indicated significant difference ($P < 0.05$)

Additives	Heated potato amylose (mg / L)	Raw potato amylose (mg / L)
Negative control	187.04 ± 10.36 A	43.03 ± 1.10A
Cell lysate		
B342	- 6.29 ± 0.66 B	-10.35 ± 1.95 B
B344	- 4.45 ± 0.94 B	-13.13 ± 3.49 B
Purified Protein		
B342	- 6.68 ± 0.55 B	4.63 ± 4.61 B
B344	- 2.76 ± 1.25 B	0.76 ± 3.43 B

7.3.4. Biochemical characterisation of α- (1→4)-branching activity of *glgB* cloned from swine intestinal bacteria

To investigate the α- (1→4)-branching activity of *glgB* on different substrates, raw potato amylose and heated potato amylose were digested by B342 / B344 clone expressed protein (Table7.1). The initial concentration of amylose added was standardized to 200 ± 5 mg / L. Heat treatment increased 87% of iodine-binding amylose detected in negative control. This was possible related to the double helix structure of potato amylose which can be destroyed by heat treatment with excessive water resulting in more iodine-amylose complex [373,374]. Heated amylose was completely digested by B342 / B344 clone expressed *glgB* while raw potato amylose was still partially digested. Compared to negative control, significant reduction of iodine-binding amylose in raw potato amylose was observed by adding cell lysates and purified proteins. But no significant

difference was observed between cell lysates and purified proteins indicating the purified proteins are the key component reducing iodine-binding amylose in potato amylose.

7.4. Discussion

Metagenomic sequencing has facilitated the prediction of microbial composition and metabolic functions of environmental niches [375]. But it is challenging to translate the presence of a specific gene in a certain niche into prediction of confirmed metabolic activity, which still requires extra biochemical validation for the specific biological process [366,376]. In our previous prediction of microbial degradation of starch in swine intestine, *Firmicutes* harbored a high abundance of α- (1→4)-branching enzyme *glgB* dominating the extracellular degradation. This branching enzyme was suggested to increase the accessibility of insoluble starch by introducing more α- (1→6)-chains [367]. The fact is both glycogen branching enzymes and starch branching enzyme catalyze the formation of α- (1→6)-glyosidic bonds on amylose and amylopectin. The difference between the action modes of glycogen branching enzymes and starch branching enzymes still should be carefully distinguished [377,378]. That is glycogen branching enzymes tend to produce more (8-9%) but shorter branches (10-12 glucose residues) in glycogens while starch branching enzymes usually generate less (3.5%) but longer branches (20-23) in amylopectin [379,380].

In this study, we confirmed that gene cloning and protein purification retained the activity of *glgB* on amylose. The different reductions of iodine-binding amylose in raw and heated potato amylose after digestion reflected the activity of *glgB* on α- (1→4)-amylose and suggested the needs for potent branching enzyme to increase the accessibility of raw potato amylose to amylase. In addition, the iodine-binding capacity of amylose increases with the average degree of polymerization [381]. But the reduction of iodine-binding amylose treated by *glgB* cloned cell extracts exceeded the average number of untreated controls, which positively indicated the release

of iodine-binding amylose by *glgB* treatment and the reasonable length of the generated chain residues. But the introducing points, the number and length of α- (1→6)-branches still need further identification.

7.5. Conclusion

It's promising to obtain potent metabolic enzyme from intestinal bacteria based on bioinformatic prediction, which could also promote a better understanding of metabolic process in animal intestine. Gene cloning *glgB* gene from swine intestinal bacteria successfully retained its activity on α- (1→4)-amylose but the precise structure of end products still need further validation.

Table S7.1. Nucleotide sequences of glgB gene clone B342 and B344

Target	Sequence (5' → 3')
B342	ATGACAACTGTAGAAAAGAAAACACAAAATGCGCTTCCCTTTCCGGAAAAAATAACAGAATATGACCAGTATCTGTTTGCGCAGGCTA CAAACTACGACATCTACAACAAGCTTGGAAGCCATGTAACTGTAAACAATGGTGAAAAGGGTGTTTATTTTGCCGTATGGGCACCTAA GGCTAAGGCTGTAAGTCTGGTAGGCAACTTTAACAACTGGGATGGCTCTAAAAACCCAATGACCAGAAATGAGCCAAGTGGTATCTG GGATATTTTTGTGCCTGGACTTGATGTAGGTGAGGTTTATAAATATCAGATTAAGACCTGGGATGACCGTATTTTAATGAAGGCTGAC CCATATGCCAATTCTAACGAGCTTCGTCCAAACAACGCCTCTGTAGTTTCTGACATTTCACATTTTAAATGGTCAGATGCCAAGTGGATT ACAAACCGCAGAAAAACTGATATTAAAAAGGCACCAATGGCTATCTATGAAGTACATATCGGTTCATGGAGACGTCACAAGGAAGTT GGCGAAAATGGCACTGATTTCTATAATTATCGTGAATTTGCCCATGAAATAACTGATTATGTAAAACAGATGGGCTATACTCATATTGA ATTAATGGGTATTGCTGAACATCCATTTGATGGCTCATGGGGCTATCANGTAACTGGCTATTACGCACCTACTTCAAGATATGGAACA CCAGAAGATTTCATGTACATGATTAACTATCTGCATGAGCATAAGATTGGTGTTATCTTAGACTGGGTTCCTGCACATTTTCCAAGAGA TGGTCATGGTCTGGCTAAATTTGATGGTACAGCTGTCTATGAATATGCCGATCCTAAGAAGGGAGAACATCCTGACTGGGGTACAAT GATTTTTGATTTCGGCAAGAATGAAGTTAAGAACTTCCTGATTGCCAATGCACTATTCTGGATTGAAAAGATGCATCTGGATGGTTTGA GAGTTGATGCCGTTGCCTCAATGTTATATCTTGATTATGGCAAACAGACTGGACAGTGGATTGCCAACAAATACGGCGGCAACAAGA ATCTTGAGGCTATAGAATTCTTTAAACACTTAAATACCTGTATTGTTGGCCGTAATCCTGGCACCATGATGATAGCCGAGGAATCAACC GCCTGGCCAAAGGTTACCGGCGATCCTAAAGATGATGGTCTGGGCTTTACATTCAAGTGGAATATGGGCTGGATGCATGACTTCCTG GATTATATGAAGCTTGATCCATTATTCAGAAAGTTTAATCATAACAAGATGACTTTCTCCATGATGTATGCTTATTCTGAGAATTTCATT CTGGTACTTTCACATGATGAAGTTGTACACCTGAAGTGTTCAATGCTGAATAAAATGCCTGGCTATCCAGCTGATAAATTCAAGAATCT GAAAGCTGCCTACGCATTTATGATTGGTCATCCTGGCAAGAAACTGCTGTTTATGGGTCAGGACTTTGGACAGCTCAGAGAATGGTCA GAGGAAAGAGAACTTGACTGGTTCCTGTTAGAAGAAGCTGAACACAAGAACCTGCAGACTTACTATGCAGATCTGCTGCACATCTAT AATTCTTATCCGGTTTTATATAACAGTGACTATACACCAGATGGCTTTAAGTGGATTAATGCCGACGATGGTGACAGATCAATCTTCTC GTTTGTAAGACTCTCGCCTACCAAAAAGAAGAATCTCCTGTTTGTAGTAAACTTCACACCAATGGAGAGAACTGACTACAGAGTAGGT GTACCAAACAGAAGAACCTATAAGCTGATTCTGGATTCTGAAGATCCTAAATATGGTGGAAGTGCTCCAGAAGATAAGCAGAAGCTC TATAAGGCCGAAAAGAAGGAATGTGACAATCAGAAGTTCTCATTTGCCTATTCCTTACCAGCCTATGGCGTTGCGGTATTTGAATTCTA A
B344	ATGACAACTGTAGAAAAGAAAACACAAAATGCGCTTCCCTTTCCGGAAAAAATAACAGAATATGACCAGTATCTGTTTGCGCAGGCTA CAAACTACGACATCTACAACAAGCTTGGAAGCCATGTAACTGTAAACAATGGTGAAAAGGGTGTTTATTTTGCCGTATGGGCACCTAA GGCTAAGGCTGTAAGTCTGGTAGGCAACTTTAACAACTGGGATGGCTCTAAAAACCCAATGACCAGAAATGAGCCAAATGGTATCTG GGATATTTTTGTGCCTGGACTTGATGTAGGTGAGGTTTATAAATATCAGATTAAGACCTGGGATGACCGTATTTTAATGAAGGCTGAC CCATATGCCAATTCTAACGAGCTTCGTCCAAACAACGCCTCTGTAGTTTCTGACATTTCACATTTTAAATGGTCAGATGCTAAGTGGATT ACAAACCGCAGAAAAACTGATATTAAAAAGGCACCAATGGCTATCTATGAAGTACATATCGGTTCATGGAGACGTCACAAGGAAGTT GGCGAAAATGGCACTGATTTCTATAATTATCGTGAATTTGCCCATGAAATAACTGATTATGTAAAACAGATGGGCTATACTCATATTGA ATTAATGGGTATTGCTGAACATCCATTTGATGGCTCATGGGGCTATCANGTAACTGGCTATTACGCACCTACTTCAAGATATGGAACA CCAGAAGATTTCATGTACATGATTAACTATCTGCATGAGCATAAGATTGGTGTTATCTTAGACTGGGTTCCTGCACATTTTCCAAGAGA TGGTCATGGTCTGGCTAAATTTGATGGTACAGCTGTCTATGAATATGCCGATCCTAAGAAGGGAGAACATCCTGACTGGGGTACAAT GATTTTTGATTTCGGCAAGAATGAAGTTAAGAACTTCCTGATTGCCAATGCACTATTCTGGATTGAAAAGATGCATCTGGATGGTTTGA GAGTTGATGCCGTTGCCTCAATGTTATATCTTGATTATGGCAAACAGACTGGACAGTGGATTGCCAACAAATACGGCGGCAACAAGA ATCTTGAGGCTATAGAATTCTTTAAACACTTAAATACCTGTATTGTTGGCCGTAATCCTGGCACCATGATGATAGCCGAGGAATCAACC GCCTGGCCAAAGGTTACCGGCGATCCTAAAGATGATGGTCTGGGCTTTACATTCAAGTGGAATATGGGCTGGATGCATGACTTCCTG GATTATATGAAGCTTGATCCATTATTCAGAAAGTTTAATCATAACAAGATGACTTTCTCCATGATGTATGCTTATTCTGAGAATTTCATT CTGGTACTTTCACATGATGAAGTTGTACACCTGAAGTGTTCAATGCTGAATAAAATGCCTGGCTATCCAGCTGATAAATTCAAGAATCT GAAAGCTGCCTACGCATTTATGATTGGTCATCCTGGCAAGAAACTGCTGTTTATGGGTCAGGACTTTGGACAGCTCAGAGAATGGTCA GAGGAAAGAGAACTTGACTGGTTCCTGTTAGAAGAAGCTGAACACAAGAACCTGCAGACTTACTATGCAGATCTGCTGCACATCTAT AATTCTTATCCGGTTTTATATAACAGTGACTATACACCAGATGGCTTTAAGTGGATTAATGCCGACGATGGTGACAGATCAATCTTCTC GTTTGTAAGACTCTCGCCTACCAAAAAGAAGAATCTCCTGTTTGTAGTAAACTTCACACCAATGGAGAGAACTGACTACAGAGTAGGT GTACCAAACAGAAGAACCTATAAGCTGATTCTGGATTCTGAAGATCCTAAATATGGTGGAAGTGCTCCAGAAGATAAGCAGAAGCTC TATAAGGCCGAAAAGAAGGAATGTGACAATCAGAAGTTCTCATTTGCCTATTCCTTACCAGCCTATGGCGTTGCGGTATTTGAATTCTA A

Chapter 8. General discussion and conclusion

8.1. Quantitative profile of virulence factors and virotype identification of ETEC in pig

The diversity of virulence factors of swine-associated ETEC and their hybrid virotypes at different age and geographical locations contribute to the unpredictability of ETEC diagnostics and prevention [50]. In the longitudinal surveillance of ETEC virulence factors in weanling pigs, the high-resolution pathogenicity was determined by multiplex HRM-qPCR analysis. By simultaneously determining five swine-associated fimbriae, the identification of dominant fimbriae type and the presence of other fimbriae types could be detected in the same reaction (Chapter 3). Combining with the quantification of enterotoxins, the diagnosis of virulence determinants could provide the basis for the development of ETEC vaccines and fimbriae-targeted treatment for weanling pigs [50,197].

The virulence of ETEC is dependent on the presence of toxins and adhesion factors in the same genome; therefore, despite the comprehensive determination of virulence factors in swine feces, the virotype of ETEC strains involving in PWD still cannot be characterized by PCR-based assays with gDNA as template. One possibility is to use multiplexed single intact cell droplet digital PCR (MuSIC ddPCR), which uses whole cells as template and thus enables the determination of co-occurrence of fimbriae and enterotoxins in the same cell [382–384]. MuSIC ddPCR has been successfully applied for EHEC detection in food enrichment samples, by detecting genetic targets (*stx* and *eae*) in the same bacterial cell to distinguish EHEC from false positives. [382]. Combining the quantification of all the virulence factors with the further diagnosis of ETEC virotype would greatly broaden the understanding of ETEC pathogenicity in pigs and thus improve the prevention of ETEC in swine production.

8.2. Swine as source of fermentation organisms for cereal fermentations

Gut microbiota is the collectivity of microbes living in the gastrointestinal tract, with the dominance of bacteria and presence of archaea. The dynamic equilibrium of gut microbiota is mainly influenced by host physiological conditions, antibiotics, diet, and particularly dietary carbohydrates. [292,294,295,385]. These factors alone or together determine the resources, stress and disturbance in intestinal tract thus alter microbial compositions and thus contribute to dramatic difference between the forestomach microbiota and large intestinal microbiota [294,298,385,386]. The low gastric pH prevents that acid-sensitive microorganisms colonize the foregut. *Lactobacillus* dominates the microbiota in swine proximal gut by adhering to the surface of non-secretory sites, and continuously inoculates passing digesta [230,387]. Sourdough fermentation initially start with mixture of flour, water and starter cultures and gradually develop into relatively stable ecosystem by continuous propagation. Lactic acid bacteria, possible together with yeast are the major fermenters of sourdough and lactobacilli are typically the dominant species in sourdoughs. With respect to the pH, the regular supply of nutrients and the temperature, the swine forestomach resemble sourdough fermentations. Despite the vast difference in complexity and selection pressure of sourdough fermentation and gut microbiota, the metabolic traits required by survival and adaptation in sourdough coincide with that is needed for colonization in swine intestine [148,388,389].

The essential of adaptations is mainly related to microbial competitions, both for the sourdough fermentation and probiotic application. Swine-adapted *L. reuteri* outcompeted ETEC in ileum or caecum by exclusively occupying colonizing sites but did not decrease the abundance of luminal *Clostridium* Cluster I. The production of bacteriocin is another characteristic of *L. reuteri* for interference competition, which widely exists in natural ecosystems [390]. Reutericylin production

did not inhibit Gram-positive *Clostridium* Cluster I in swine intestine but reutericyclin contributed to the competition against *L. sanfranciscensis* thus aid in the stable persistence of *L. reuteri* in sourdough. Host adapted *L. delbrueckii*-group species and *L. reuteri* also populate in type II sourdoughs and benefit the quality of sourdough and sourdough bread. These common points enable the possibility to translate the ecological interactions in swine stomach to sourdough fermentation thereby facilitating the selection of sourdough starter cultures. The shared selection pressure in cereal fermentations and the swine stomach also facilitates the use of host-adapted lactobacilli in feed fermentations to supply probiotic strains.

8.3. Selection criteria for swine probiotic requires further linkage between host adaptation of strains and host immune responses

The content of *Clostridium* cluster I decreased equivalently in pigs fed with *L. reuteri* and *L. fermentum* / *L. casei* while only inclusion of *L. reuteri* reduced the abundance of total *E. coli* and enterotoxins. The release of enterotoxin in pigs is mediated by swine-specific fimbriae adhering to intestinal epitheliums [50,60,264]. Thus, the divergent observations could be translated to that host-adapted *L. reuteri* effectively inhibited the colonization of ETEC without fimbriae-type specificity and reduced the release of toxins in swine intestine. In addition, the better survival of *L. reuteri* during intestinal transit also supported the higher competitiveness of host-adapted strains in swine intestine. If we infer that the reduction in *Clostridium* Cluster I is equivalently due to immunomodulatory effects of *L. fermentum* / *L. casei* and competitive exclusion of *L. reuteri*, these results perfectly support the host-adaption-based selection criteria for swine probiotics. But the major limitation of this thesis is the lack of information about immune responses induced by these lactobacilli in pig. Moreover, the molecular mechanisms involving in divergent immune responses induced by autochthons and allochthons in pigs were not so well explained as that in

human and mice. Therefore, these findings support the selection criteria for probiotics based on their host adaption but also requires more validation of the corresponding immune responses in pigs.

8.4. Limitations and future directions

As discussed above, one of the major limitations of this study is the lack of host immune response to host-adapted and nomadic lactobacilli. According to immunological studies in mice and human, allochthonous probiotics are more likely to induce the overexpression of inflammatory cytokines and skew the immunity towards inflammation. But this conclusion cannot be translated to pigs due to insufficient supports from animal studies. Most of probiotic studies relied on IPEC-J2 / Cao2 cell lines based *in vitro* assays rather than animal study to evaluate the immunomodulation of probiotics. Therefore, further immunological research on pigs is a crucial piece to fill the gap and support the selection criteria of probiotics suggested in this thesis.

Another limitation is the absence of direct evidence of exclusive colonization of host-adapted lactobacilli. This study proposed host-adapted lactobacilli possess higher competitiveness to outcompete attach sites thus reduce ETEC adhesion in swine intestine. Although better persistence and pathogen inhibition of host-adapted lactobacilli were observed in this thesis, the direct proof showing the link between lactobacilli colonization and reduced ETEC attachment was still missing. Florescent *in situ* hybridization (FISH) is one possibility to provide solid evidence by quantifying the attached ETEC in swine intestine before and after probiotic intervention.

In addition, biochemical characterization of Glgb branching enzyme was only preliminarily confirmed by the reduction of amylose in this study. Based on the bioinformatic prediction in Chapter 6, this enzyme converted unbranched starch into highly branched structures and thus increased their solubility and accessibility to glycosidases in swine intestine. Moreover, fewer but

longer branches were introduced by glgB starch branching enzyme into insoluble amylose which is distinguishable from glycogen branching enzymes formatting more and shorter branches in glycogens. Thus, the introducing points, the number and length of α- (1→6)-branches introduced by glgB branching enzymes still need further identification.

8.5. Conclusion

In summary, host adaption played a central role in competitiveness of probiotics strains in swine intestine thus exerted divergent effects on pathogenic and commensal microbiota (Chapter 4 and 5). According to the specific purpose in swine production, using the probiotic strains with desired properties is the key criteria for selection of swine probiotics (Chapter 2). Precise determination of pathogenesis relies on the development of detection methods and is necessary for effective prevention of disease. Combining probiotic application and pathogen detection is an efficient way to reduce risk of infection by intestinal pathogens in current swine production systems and thus reduced usage of antibiotics (Chapter 3). The reconstruction of gut microbial metabolism improved the understanding of carbohydrate fermentation in the swine intestine also enabled the development of feeding models with higher feed efficiency and better pathogen control for weanling pigs (Chapter 6 and 7).

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