Fermented Whey Permeate for Piglets

As a strategy to reduce Post Weaning Diarrhoea

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FERMENTED WHEY PERMEATE FOR PIGLETS

As a strategy to reduce Post Weaning Diarrhoea

Ph.D. Thesis

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National Veterinary Institute

Technical University of Denmark

Table of Contents

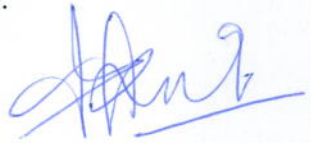
Preface

This thesis represents part of the requirements for the Ph.D. degree at the Technical University of Denmark (DTU). The work was carried out at the National Veterinary Institute, DTU and the Department of Animal Health, Welfare and Nutrition, Faculty of Agricultural Sciences, University of Aarhus. My supervisors were Dr. Lars Mølbak and Prof. Mette Boye from DTU-VET and Dr. Bent Borg Jensen from University of Aarhus. The work was financed by DTU, Danish Meat Council and Arla Foods for a period of three years.

The thesis consists of two different types of material. The first section provides an introduction to the general area that is related to the study. The obtained results are discussed in the light of the present knowledge in the area. The second part consists of four papers that are either submitted or in preliminary forms to submit for publications in international journals. These papers provide detailed descriptions of the performed works throughout the PhD project.

I'd like to express my gratitude to a number of people, who have helped and supported me in various ways during my three years at DTU. **Lars Mølbak** has been a great supervisor who has been involved in many scientific discussions, providing insightful ideas, technical expertise, challenges and motivation to carry out the project. **Bent Borg Jensen** has been resourceful especially in carrying out piglet trials, which are crucial parts of this project. He and his staffs in Foulum have always been accommodating and helpful during my visits. **Mette Boye** has been dependable in helping me to communicate with the PhD administration, especially during the final 6 months of my study. **Kirstine Schou** who helped by listening and providing advice during my ups and downs at the office and laboratory. All the technicians at the Microbial Ecology group, who trained me in the lab, and created a good atmosphere to work in the group. **Prof. Cheng He** and **Changbo Ou** who helped me during my stay and guide me in performing experiments and extractions at the Chinese Agriculture University.

Last but most importantly, **my family**, from whom I had to live apart during most of these three years, for believing in me and never cease loving me.



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Summary

The intestine is an essential compartment of the gastrointestinal tract (GIT). It is a major site of digestion, nutrient absorption and hydro-mineral exchange homeostasis, harbouring a complex microbiota and a highly evolved mucosal immune system. Interactively, all these aspects of GIT physiology, microbiology and immunology contribute to the so-called "gut health balance".

Weaning places piglets in a high risk situation. The pigs' gut health balance is challenged by the different stress factors, including separation from the sow and an abrupt change from milk to a diet based on cereals. Consequently, the young animals become susceptible to infections by different pathogens, which may lead to post weaning diarrhoea (PWD).

The challenge is to protect young pigs from developing PWD without using antibiotics as growth promoters, especially since the practice is banned in Europe(Casewell et al., 2003). Changing the composition of the weaners diet, including the addition of probiotic, prebiotic, or phytobiotic improve growth performance, resistance to diarrhoea and sometimes manipulate the composition of the microbiota and its metabolic activities (Pierce et al., 2005; Kommera et al., 2006; Canibe and Jensen, 2007; Konstantinov et al., 2008)(Pierce et al., 2005; Canibe and Jensen, 2007; Canibe et al., 2007; Molbak et al., 2007).

Whey permeate, especially its lactose content, is one of the alternative nutrient sources which benefit piglets (Pierce et al., 2006). The first part of this thesis tried to answer the hypothesis that the lactose content in whey permeate improves growth performance in piglets after weaning without disturbing the gut health homeostasis. Analyses on gut samples including microscopic observations on the mucosa layer morphology, measurements of short chain fatty acids contents and composition of microbiota communities indicated that post weaned piglets indeed are capable of metabolizing lactose and maintain gut health. These observations were confirmed by improved conversion rate of feed to average daily gain even at inclusion of low level of whey permeate.

Concurrently, addition of probiotic seems to help to balance the gut microbiota, limit the colonisation of coliform bacteria, which may help to prevent severe PWD in piglets (Taras et al., 2006; Konstantinov et al., 2008). However, the responses of piglets to probiotic treatment are strain dependent and often inconsistent (Simon. 2010; Kenny et al., 2011). It would be ideal to obtain selected strains which not only show potential as probiotic for piglet

application, but also are capable to proliferate in whey permeate. The second and third parts of this thesis were performed to elucidate these questions (1) whether whey permeate can be used as a medium to proliferate selected lactic acid bacteria which show potential as probiotics and (2) whether selected lactic acid bacteria, when added as inoculum to ferment whey permeate, reduce PWD in infected piglets.

Probiotic selections for pig applications follow similar recommendations available for the human application (FAO/WHO, 2001). We found that only few lactic acid bacteria are able to proliferate in whey permeate without supplementations and maintain their probiotic potential. Our final selections consist of 3 *Lactobacillus plantarum* isolates, 1 *L. rhamonsus* isolate and 2 isolates from the genus *Weissella*

One of the *L. plantarum* isolates (*L. plantarum* 65) and 1 isolate from the genus *Weissella* (*W. viridescens* 19) were used as inoculums to ferment whey permeate. The fermented whey permeate product was mixed with basal weaners diets and fed to piglets challenged with *E. coli* F4. The infection model helped us to identify the potential of the fermented product to minimize diarrhoea from post weaned piglets. Our experiments confirmed that fermenting whey permeate with the potential probiotic *W. viridescens* reduced diarrhoea frequency, improved feed intake and production of butyric acid in colon and at the same time increased the abundance of Firmicutes in colon.

Post weaning diarrhoea is a global challenge to the pork industry. In China, one of practices to prevent and cure PWD is by the addition of phytobiotics (Kong et al., 2007; Ding et al., 2011). (Kong et al., 2007; Ding et al., 2011)The last part of the project dealt with evaluation of Chinese Herbal Medicine, in the form of *Fructus mume* and *Ziziphi spinosa* semen ethanolic extracts, in inhibiting PWD-relevant *E. coli* F4 and measuring how these extracts regulate innate immune responses *in vitro*. The experiments revealed that indeed extracts of *Fructus mume*, and its mixture with *Ziziphi spinosa* semen, are bactericidal against *E. coli* F4. Furthermore, measurements of cytokine expressions on intestinal porcine epithelial cell line (IPEC-J2) revealed that *Fructus mume* regulates immune response by down regulating IL-18 and TNF-α and by upregulating TLR-4.

In conclusion, the works in the present thesis provides knowledge that fermenting whey permeate with a selected probiotic may be an economical yet efficient approach in reducing diarrhoea and helping post weaned piglets to regain their health gut balance. Furthermore,

application of *Fructus mume* may also be another alternative for PWD management. However, an in vivo study to confirm the efficacy in pigs is required.

Dansk Resumé

Tarmen udgør en essentiel del af mave-tarmsystemet (GIT) hos grise. Tarmen er et vigtigt organ for fordøjelse, næringsabsorption og mineral balance. I tarmen findes en kompleks mikrobiota og et højt udviklet immunsystem. Disse forskellige aspekter af tarmens fysiologi, mikrobiologi og immunologi er medvirkende faktorer til den samlede "tarmsundhed".

Ved fravænning er der en høj risiko for, at grisene kan få fravænningsdiarre (PWD). Tarmsundheden hos grisene udfordres af forskellige stress faktorer som adskillelse fra soen og en brat ændring fra soens mælk til foder. Som konsekvens heraf vil det unge dyr være mere modtageligt for infektioner forårsaget af forskellige sygdomsfremkaldende mikroorganismer, hvilket kan være medvirkende til PWD.

Udfordringen består i at beskytte grisene mod PWD uden brug af antibiotiske vækstfremmere, hvis brug ikke er tilladt i Europa (Casewell et al., 2003). Ved at ændre sammensætningen af grisenes foder omkring fravænning ved tilsætning af for eksempel probiotika, præbiotika eller phytobiotika kan daglig tilvækst forbedres og modtageligheden for infektioner formindskes. Disse fodertilsætninger kan også have indvirkning på sammensætningen af tarmens mikrobiota (Pierce et al., 2005; Kommera et al., 2006; Canibe and Jensen, 2007; Konstantinov et al., 2008)(Pierce et al., 2005; Canibe and Jensen, 2007)(Canibe et al., 2007; Molbak et al., 2007).

Vallepermeat, og specielt laktose indholdet i permeat, er en af de alternative fodertilsætninger, som kan være l gavnlig for grise (Pierce et al., 2006). I den første del af denne afhandling undersøges hypotesen, hvorvidt laktose indholdet i permeat kan forbedre daglig tilvækst af grise efter fravænning uden at forstyrre tarmsundheden. Analyse af tarmprøver inkluderer mikroskopiske undersøgelser af morfologien af tarmslimhinden, bestemmelse af indholdet af kortkædet fedtsyrer og sammensætningen af mikrobiotaen. Disse undersøgelser viste, at fravænningsgrise er i stand til at metabolisere en høj koncentration af laktose og stadig opretholde tarmsundheden. Dette blev yderligere bekræftet ved forbedring af omsætningen af foder til daglig tilvækst selv ved tilsætning af små koncentrationer af permeat.

Tilsætning af probiotika til foderet kan hjælpe til at opretholde tarmsundheden herunder en "sund" mikrobiota. Probiotika kan også reducere koloniseringen med koliforme bakterier, som derved kan medvirke til minimering af alvorlig PWD i fravænningsgrise (Taras et al., 2006; Konstantinov et al., 2008). Men udbyttet af tilsætning af probiotika er afhængig af de

specifikke bakteriestammer og ofte er resultaterne af denne fodertilsætning varierende. (Simon. 2010; Kenny et al., 2011).

Det vil være ideelt at opnå bakteriestammer, som både viser potentiale som probiotika til fravænningsgrise, men som også er i stand til at opformeres i permeat. Formålet med det andet og tredje manuskript i denne afhandling var at opnå viden om (1) hvorvidt permeat kan bruges som vækstmedium for udvalgte mælkesyrebakterier med potentiale som probiotika og (2) hvorvidt de selekterede mælkesyrebakterier, tilsat permeaten, er i stand til både at fermentere permeaten og samtidig minimere PWD i *E. coli* inficerede fravænningsgrise.

Probiotika til brug i grise følger de samme anbefalinger som probiotika til humant brug (FAO/WHO, 2001). Vi fandt, at kun få mælkesyrebakterier er i stand til at fermentere permeat uden tilsætning af andre næringsstoffer og samtidig have probiotisk potentiale. Vores endelige selektion af mælkesyrebakterier bestod af tre *Lactobacillus plantarum* isolater, et *L. rhamonsus* isolat og to isolater fra familien *Weissella*

Et af *L. plantarum* isolaterne (*L. plantarum* 65) samt et isolat fra familien *Weissella* (*W. viridescens* 19) blev brugt som inokulum for fermentering af permeat. Det fermenterede permeat blev blandet med standardfoder til fravænningsgrise og brugt som foder til grise inficeret med *E. coli* F4. Den eksperimentelle infektionsmodel i fravæningsgrise undersøgte potentialet af det fermenterede produkt til minimering af PWD. Forsøget viste, at permeat fermenteret med den potentielle probiotiske bakterie *W. viridescens* reducerede diarre, forbedrede foderudnyttelse og produktion af smørsyre i tyktarmen. Antallet af bakterier tilhørende slægten Firmicutes blev samtidig forøget i tyktarmen.

Fravænningdiarre er en global udfordring for svineindustrien. I Kina udnyttes blandt andet phytobiotika som fodertilsætning til minimering af PWD (Kong et al., 2007; Ding et al., 2011). Det sidste manuskript i denne afhandling undersøgte traditionelt kinesisk medicin i form af ekstrakter af *Fructus mume* og *Ziziphi spinosa* til brug for minimering af PWD forårsaget af *E. coli* F4. Det blev yderligere undersøgt, hvordan disse ekstrakter indvirkede på det innate immunsystem i en *in vitro* cellemodel. Forsøgene viste, at ekstrakt af *Fructus mume* alene eller i blanding med *Ziziphi spinosa*, virker baktericidt mod *E. coli* F4. Analysen af det innate immunsystem ved brug af den porcine intestinale cellelinie IPEC-J2 viste, at *Fructus mume* regulerer immunresponset ved at nedregulere IL-18 og TNF-α og opregulere TLR-4.

Konklusionen på det eksperimentelle arbejde, der præsenteres i denne afhandling, er at permeat fermenteret med udvalgte probiotiske bakterier kan udgøre en potentiel økonomisk rentabel og effektiv metode til minimering af fravænningsdiarre og medvirke til opretholdelse af grisenes tarmsundhed. Udnyttelse af traditionelt kinesisk medicin i form af fodertilsætning af ekstrakt af *Fructus mume* kan udgøre et andet alternativ for kontrol af PWD, men fremtidige *in vivo* forsøg vil vise dette.

1. Introduction

One of the motivations to introduce probiotic in pork production is the challenge of controlling post weaning diarrhoea (PWD), especially since the banning of antibiotic usage (Lalles et al., 2007a; Lalles et al., 2007b; Simon. 2010). The mechanisms of how probiotic confer health benefits to pigs continue to be better understood. The focus of in-feed probiotic applications is to promote gut health, which is observed from measureable improved growth performance, efficient feed to growth conversion, reduction of diarrhoea symptoms and in some cases reduction of pathogens and improved microbial diversity (Konstantinov et al., 2008; de Lange et al., 2010; Krause et al., 2010). Gut health among post weaned piglets translate to an increased production yield for the farmer. It is undeniable that the effects are strain dependent with strong interplay between the administrated bacteria and the host' resident microbiota (Bhandari et al., 2008; Simon. 2010).

Likewise, in-feed addition of whey permeate improves growth performance and gut health among post weaned piglets (Pierce et al., 2006; Pierce et al., 2007; Naranjo et al., 2010). It is hypothesized that the abundance of lactose in whey permeate support the proliferation of lactic acid bacteria (Hugenschmidt et al., 2010; Panesar et al., 2010). It is widely known that the majority of commercial probiotic strains belong to the group lactic acid bacteria (Lalles et al., 2007). Indeed, it may be possible to select novel strains which not only propagate quickly in whey permeate, but also exhibit probiotic traits. The combination of lactose and viable potential probiotic in fermented whey permeate may be beneficial to promote gut health among post weaned piglets.

Escherichia coli serogroups O149 and O138 are the most common pathogens causing post weaning diarrhoea outbreaks (Frydendahl. 2002). An experiment which includes infecting piglets with one of these serogroups, provides a controlled model to asses responses from post weaned piglets after receiving whey permeate, fermented by pre-selected probiotics.

The purpose of the present PhD project was:

- To determine the effects of whey permeate as in-feed additions on the growth performance, gut morphology, and colonic microbiota of healthy post weaned piglets (paper I)

- To screen lactic acid bacteria, from various natural sources, based on its ability to proliferate in unsupplemented whey permeate and the *in vitro* probiotic characteristics (paper II).
- To establish an infection model by which to asses the effect of whey permeate with or without fermentation by selected probiotic strains on gut health and growth performance (paper III).
- To evaluate the effect of ethanolic extract *Fructus mume* and *Ziziphi spinosa* semen in inhibiting *E. coli* and in modulating innate immune response *in vitro* (paper IV).

2. Pig gastrointestinal tract (GIT) development and health

The gastrointestinal tract (GIT) of pig is a complex environment. It is part of the digestive tract which main function are to digest food by various digestive juices and enzymes, facilitate absorption of nutrients by the host, and remove unabsorbed food components (Walthall et al., 2005). As the name implies, GIT is built as an open ending system consisting of distinctive parts: stomach (gastro--), and small and large intestines.

Researchers consider postnatal development of the GIT into three phases: the birth and early suckling phase, the suckling phase, and the weaning phase (Walthall et al., 2005). Specifically among newborns and around the time of weaning, pigs' gut rapidly changes in size, has high protein turnover rates, undergoes rapid changes in microbiota and quickly alters its digestive and immune functions (Bailey et al., 2005; Lalles et al., 2007a; de Lange et al., 2010) before stabilizing into matured GIT system (Walthall et al., 2005).

2.1 Microbiota

2.1.1 Determination of microbial communities

The microbial communities of the gastrointestinal tract are not fully understood due to the inadequacy of classical, culture-dependent microbiological methods. More than two decades ago, most efforts were put into characterizing the intestinal microbiota of pigs by using microbiological methods based on culturing and phenotypic analysis of the isolates (Robinson et al., 1981; ALLISON. 1989). These studies showed that the majority of the culturable bacteria are Gram-positive, strict anaerobic streptococci, lactobacilli, eubacteria, and clostridia, while *Bacteroides* dominates the Gram-negative group. No information of community changes to environmental perturbations could be obtained because culture-based methods are very time-consuming, thereby limiting the number of samples that can be processed.

Within the past 20 years, we have witnessed an immense growth and development in culture-independent techniques to evaluate microbial communities. Detailed information of the microbial community composition in pigs can be gained from the phylogenetic analysis of 16S ribosomal RNA (rRNA) gene sequences obtained directly from samples by PCR amplification, cloning, and sequencing (Leser et al., 2002). Some of the other techniques which also utilised amplification of 16S rRNA gene sequenes to analyse microbial communities in animals including in pigs are denaturing gradient gel electrophoresis (DGGE)

(Konstantinov et al., 2006), terminal restriction length polymorphism analysis (T-RFLP) (Krause et al., 2010), next-generation sequencing (Kim et al, 2011) and DNA microarray (Zoetendal et al, 2008).

2.1.2 Next generation sequencing

More recently, next generation sequencing was included in microbiota community analyses of samples from different sources(Neufeld et al., 2004). The advantage of this new technique is the possibility to determine the sequence data from amplified single DNA fragments, avoiding the need for cloning DNA fragments (Ansorge. 2009). However, the challenge of having the PCR-related bias remains (Dohm et al., 2008; Haas et al., 2011).

Typically, next generation sequencing is carried out by pyrosequencing on a 454 Genome Sequencer FLX machine (http://454.com/applications/metagenomics/index.asp) or the Illumina (http://www.illumina.com/technology/sequencing_technology.ilmn) analyser. The amplicons (sequence reads) of a single variable 16S rRNA gene region are quantified and subsequently assigned to microbial phylogenies (and thence to taxonomies). The nine different variable 16S rRNA gene regions are flanked by conserved stretches in most bacteria , and they can be used as targets for PCR primers with near-universal bacterial specificity (Yu et al., 2006; Kim et al., 2011b). Indeed this approach provides less discriminatory than the full-length 16S rRNA gene, but the massively parallel sequencing of the shorter reads offer the options of obtaining either much higher coverage per sample (Schuster. 2008) or many more samples per instrument run by means of barcoding techniques (Hamady et al., 2008). The trade-off with the longer, but fewer, reads generated by traditional Sanger sequencing means a lower proportion of amplicons that can be classified at genus or species levels. In contrast, the resolution of the community composition with amplicon pyrosequencing is potentially several orders of magnitude larger than clone library sequencing, and can be achieved at a significantly lower cost (Claesson et al., 2010).

2.1.3 Microbiota of pigs

It has been estimated that approximately 10^{14} bacteria inhabit the mammalian GIT, and it had been suggested that 500 – 1000 bacterial species build up this population (Backhed et al., 2005). In balanced (homoeostasis) GI ecosystem, bacterial communities inhabit existing niches and these communities are consistently found to occupy the GI tract. Transient species do not stably colonize the GI ecosystem, but pass through the GI tract (Backhed et al., 2005; Andersson et al., 2008; Wang et al., 2011). However, the GI microbiome is dynamic and

subject to changes due to time, age, exposure to microbes and diet. Furthermore, disruptions in the gastrointestinal microbiota have been associated with compromised gut health, even diseases (Eckburg et al., 2005; Ley et al., 2005). Figure 1 showed the progression of the amount of bacteria in different parts of GIT of monogastric animal.

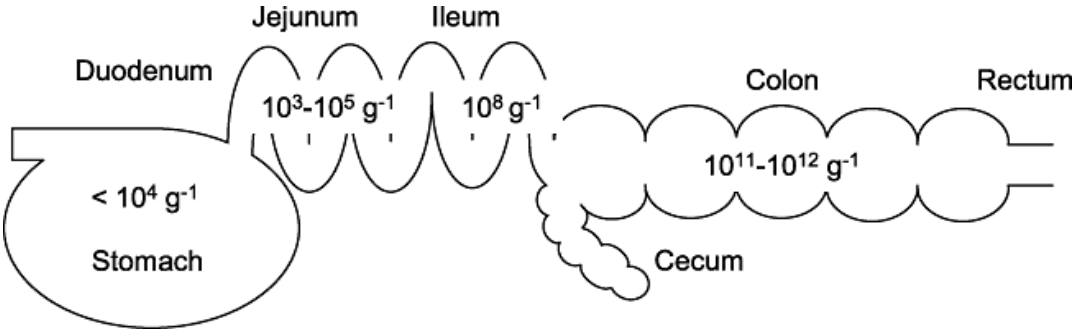

Figure 1. Schematic representation of the monogastric gastrointestinal tract. Numbers in individual sections describe the amount of bacteria per gram of intestinal digesta typically obtained in healthy individuals (Leser and Molbak, 2009).

Much less is known about the microbiome of the pig compared to human. The introduction of culture-independent techniques in studying microbial diversity in the GI tract of pigs have been limited to certain life phases, for example neonates or post weaned piglets, or focus on pigs which have received diet intervention (Leser et al., 2002; Molbak et al., 2007; Konstantinov et al., 2008). Studies to show longitudinal changes of bacterial diversity in healthy pigs across different life phases are still limited (Petri et al., 2010; Kim et al., 2011a).

The development of gut microbial community can be divided into two stages: the first stage is when piglets provide the physicochemical environment to shape early microbial community structure, and the second stage is when the microbiota becomes a "super-organism" which metabolism and living activities benefit the host. This second stage lead to the stable gut microbiome of an adult pig (Thompson et al., 2008). Colonization of the mammalian gut starts at birth. There are presumably few or no barriers to microbes from the external environment that rapidly colonize the neonates.

Based on 16S rRNA gene sequence cloned library obtained from GIT of pre weaned piglets, Clostridiaceae dominates gut microbiota community at the start (0.25 d) up to day 1, when briefly Streptococcaceae took over. Since day 5 to day 20, Lactobacilliaceae predominates the community. The bacterial succession profile is similar in the stomach, small intestine, and large intestine (Petri et al., 2010).

When compared to the other parts of GIT, large intestine, especially colon consists the largest amount of the microbiota. The preconditions which allow this abundance include the anaerobic conditions, favourable temperature, pH and slow passage of the digesta (Kidder and Manners, 1980; Mikkelsen et al., 2003). According to (Swords et al., 1993), starting at weaning, Gram-positive anaerobes were displaced by Gram negative bacteria, such as Bacteriodes.

Some of the critical roles of gut microbiota in human include vitamin and co-factors productions, metabolism of otherwise indigestible nutrients, detoxification, covering the gut surface to reduce the possibility of pathogen attacks, production of antimicrobial, maintenance of gut barrier function and promotion of anti-inflammatory responses (Kenny et al., 2011). Considering the striking similarity between pig GIT system to human, many have hypothesised that indeed, gut microbiota of pigs also plays a major role to overall well being of pigs. However, a recent comparative metagenome study among pigs at adult age (six months), showed merely 70% similarity to human metagenomes. Furthermore, the authors found swine gut metagenome clustered more closely with chicken cecal and cow rumen (Lamendella et al., 2011).

Nevertheless, there are interests in studying changes in gut microbiota during critical life periods, especially in neonates and post weaned piglets in relation to whether their health status is compromised (Konstantinov et al., 2004; Shim et al., 2005; Lalles et al., 2007b; Bhandari et al., 2008; Petri et al., 2010).

2.2 Digestive enzymes

It is understood that during the early postnatal development of pig, there are drastic and complementary changes in the levels of lactase and sucrase activity in the mucosa of the small intestine (Manners and Stevens, 1972). Immediately after birth, enterocytes, lining the villi of the small intestine produce high lactase activity which continues until 10 d after birth (Walthall et al., 2005). On the other hand, α-glucosidases and maltase are absent or present at low levels. Lactase activity decreases within 2 months of life, but the activity of other dissacharides including sucrases increases (Kidder and Manners, 1980; Adeola and King, 2006). The increase in sucrase activity is dramatic (10-fold) between 5 and 9 weeks. The author suggested the change as an adaptation to the switch of dietary carbohydrate from lactose in sow milk to predominantly starch (Adeola and King, 2006) and maturation of enterocytes (Walthall et al., 2005).

Besides brush border enzymes, pancreatic enzymes also contribute to the digesting process in GIT system of pigs. Unlike brush border enzymes which work on and around the surface of enterocytes, pancreatic enzymes work in the lumen (Hedemann and Jensen, 2004). At birth, the levels of trypsin, chymotrypsin, and amylase are lower compared to adult, but considered sufficient to hydrolyse proteinaceous moieties in sow milk(Smith. 1988). At day 3, amylase activity increases by more than 300 % while trypsin, chymotrypsin, and lipase do not change. During critical phases like weaning, pigs showed transient decreasing activities in trypsin, chymotrypsin, and amylase. Furthermore, lipase secretion start to decrease after weaning (Jensen et al., 1997).

2.3 Immune system

The development of mucosal immune system in the pig's gut environment has been reviewed (Bailey. 2009). Mucosa immune system plays primarily as a defence mechanism against potential pathogens which enter across the epithelial surface. At the same time, it also effectively controls expression of tolerance to harmless antigens.
Piglet immune system is immature at birth, and the neonate is dependent on both specific and non-specific immunity, acquired from colostrum and sow's milk (Stokes et al., 2004) as protection against enteric pathogens.

The two most crucial periods of maximum exposure to new antigens happen in the neonate immediately after birth and at weaning. There is a high chance that, in both cases, the antigenic composition of the intestinal contents change suddenly as a consequence of diet change and/ or colonisation of new bacterial strains (Bailey et al., 2005).

When does the immune system reach maturity ?

As described in (Stokes et al., 2004), the process of developing mucosal immunological architecture can be divided into four phases:
(1) Newborn, during which there are limited lymphocytes in the intestinal epithelium or lamina propria. Lymphocytes can be found as clusters in the mucosa, which subsequently will develop into Payer's patches.
(2) Early suckling period 2 weeks post-natal, when the intestine rapidly becomes colonised with lymphoid cells. These cells express the CD2 surface marker, but do not express CD4 or CD8. The Payer's patches start to organise reaching an adult-like architecture at day 10-15.
(3) Between 2-4 weeks old, the intestinal mucosa becomes colonised by $CD4^+$ T cells, mostly in the lamina propria. Few B cells start to appear.

(4) Start at an age of 5 weeks onwards, $CD8^+$ cells becomes more common in the intestinal epithelium and around the epithelial basement membrane. In the crypt area, plenty of IgA B cells are appearing. At week 7, the immune system in the intestine reaches adult- like structure (Stokes et al., 2004).

The development of an immunocompetent immune system is necessary for optimum growth. However, it is necessary to define immunocompetence by including both the ability to respond towards pathogens and the ability to tolerate food and commensal bacterial antigens (Stokes et al., 2004). Furthermore, it is directed to keep potentially harmful antigens within the lumen to allow the natural peristaltic movement and digesta flow to remove them (Stokes et al., 2004).

Intestinal health or gut health as a concept is complex. It is still difficult at present to find a consensus definition. Three main components are proposed as building blocks for "gut health" namely: the diet, the mucosa and the commensal microbiota (Conway, 1994). The mucosa consists of digestive epithelium, gut-associated lymphoid tissue (GALT) and mucus overlying the epithelium. The diet which comes into the gut from the environment arguably affect this equilibrium. Interactions among GALT, commensal bacteria, mucus and host epithelial cells form a dynamic equilibrium. The ability to adjust to feed and other external factors will ensure efficient functioning and absorption capacity of the digestive system which maintains the balance between the host, the microbiota and the intestine environment (Knudsen et al., 2012).

3. Disruption of GIT equilibrium at weaning

3.1 Microbiota shift at weaning

Piglets weaned within a farm environment experience significant changes in intestinal microbiota composition as responses to new diet and environment (Konstantinov et al., 2004; Lalles et al., 2007a). In an abrupt manner, the intestinal microbiota must ultimately develop from a simple unstable community into a complex and stable population, thus creating a challenge to 'colonisation resistance' or competitive exclusion' (Lalles et al., 2007a). Colonisation resistance is described as a health maintenance mechanism in which the gut microbiota participates in creating a barrier to prevent gut invasion by pathogenic bacteria (Stokes et al., 2004).

Specifically in the ileum, the population of lactobacilli is significantly lower among weaned piglets at age 19 days than in unweaned ones (Konstantinov et al., 2006). After the introduction of solid food post weaned, anaerobes increase in number and diversity to establish an adult-like pattern. This includes high amount of *Clostridium* (Konstantinov et al., 2006).

3.2 Compromised immune system

At weaning, the GIT of piglets is exposed to a large and diverse amount of environmental antigens which come from food and potentially pathogens. Under farm practices, piglets are weaned abruptly at an age between 3 – 5 weeks. At around this age, contrasting to at birth, the immune system has developed to a point of making active immune responses to antigenic challenge (Lalles et al., 2007b).

The epithelium undergoes biochemical and morphological changes and some authors have suggested that the changes may induce inflammation of the gut (Stokes et al., 2004). Changes in the cytokine patterns appear site specific along the gut. Transient increase of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, occur early (up to 2 d post weaning) and later reduce to pre weaning level, except for TNF-α which remains high in the ileum and colon (Pie et al., 2004). Tissue concentration of the anti-inflammatory cytokines and growth factor TGF-β transiently reduce in villi and increase in crypts of the duodenum and jejunum (Mei and Xu, 2005). Furthermore, weaning age influences the changes in the immune system.

3.4 Post weaning diarrhoea in piglets

The weaning time is a crucial period in the management of piglets during which, if not handled properly, post weaning diarrhoea (PWD) outbreaks may occur. PWD is the leading cause of serious economic losses in pig herds worldwide (de Lange et al., 2010; Vondruskova et al., 2010).

During the first 5 days after weaning, young piglets are exposed to some health related risk factors, including nutrition, etiology and indoor environment of housing being particularly implicated. Furthermore, weaning poses piglets to noninfectious stress factors which often trigger the development of gastroenteric disorders. These factors include (1) weaning age (Svensmark et al., 1989; Skirrow et al., 1997), (2) change of diet type from sow milk that provides piglets with immunoglobulins to creep feed (Bailey et al., 1992), (3) lost of appetite,

causing reduced feed intake (Bark et al., 1986; Spencer and Howell, 1989; Laine et al., 2008), (4) feed structure (Amezcua et al., 2002), (5) housing condition and hygiene (Ledividich and Herpin, 1994), and (6) inadequate feeder space per piglet in the pen (Amezcua et al., 2002).

Among infection cases, major bacterial pathogens related to PWD include *Escherichia coli* and members of the genera *Clostridium*, *Lawsonia* and *Brachyspira* (Moller et al., 1998). More specifically, enterotoxigenic *E. coli* (ETEC) serogroups are often linked to PWD. These serogroups include *E. coli* O8, O9, O20, O45, O64, O138, O139, O141, O149, and O157 (Svendsen et al., 1977; Nagy and Fekete, 2005). The most common *E. coli* serogroups associated with PWD in Denmark at present are O149 and O138 (Frydendahl. 2002). The infection by one of the *E.coli* serotypes which lead to diarrhoea outbreaks occur when the pre-dispose stress factors become unbearable. Internally within a post weaned piglet homeostatic imbalance happens during weaning: changes in the morphology (Hampson. 1986) and function of the small intestine (Kidder and Manners, 1980; Hampson and Kidder, 1986), shits in the microbiota balance of the small and large intestine (Bhandari et al., 2008; Konstantinov et al., 2008) and local inflammation in the small intestine (McCracken et al., 1999).

In Denmark, since 1998, the Danish Bacon and Meat Council, representing over 95% of Danish pig producers, agreed to retract the use of antibiotic growth promoters (AGP) gradually, included for prevention of diarrhoeal diseases in piglets (Vigre et al., 2008). This action was taken due to public concerns over the possibility of antibiotic resistance transfer from piglet microbiota to human microbiota (Casewell et al., 2003). Since 1 January 2000, all use of AGP in the Danish pig production was banned. The effect of AGP withdrawal on incidences of diarrhoea, arthritis, pneumonia, unthriving and miscellaneous diseases in 68 farrow-to-finish Danish pig farms was evaluated. The discontinuation of AGP affected the treatment on diarrhoea (Vigre et al., 2008) resulting in an increase in use of therapeutic antibiotics, especially for treating post weaning diarrhoea in piglets (Casewell et al., 2003; Vigre et al., 2008). Obviously this trend does not resonance well with the original concern that initiated the prohibition of antibiotic addition as growth promoting factor.

The challenge leads to efforts for finding alternative to AGP which would be efficient in protecting young piglets from post weaned diarrhoea. Various natural materials such as probiotics, prebiotics, alternative carbon source, organic acids, zinc and plant extracts have been tested as effective alternatives to antibiotics. To keep the content of this chapter relevant

to the overall thesis, only probiotics, alternative carbon source in the form of lactose, and plant extracts will be further explored.

4. Whey permeate

4.1 Source

Whey permeate (also called dairy product solids, deproteinized whey or modified whey) is a co-product from the production of whey protein concentrate, whey protein isolate, ultrafiltered milk, milk protein concentrate or milk protein isolate in dairy processing. Whey permeate as defined by the Reference Manual for US Whey and Lactose products (2011) covers a family of products that have a minimum of 59 percent lactose, and a maximum of 10 percent protein and 27 percent ash. Composition of permeate varies depending on the original material used and the processing involved obtaining it. Sweet whey and milk are the most common starting materials for permeate production. Whey permeate varied in its content due to variations in how each cheese manufacture performs downstream process to its whey.

4.2 Whey permeate applications

Abundant and bulky, whey permeate application and valorization is of enormous importance to the sustainability of dairy processing industries (Smithers. 2008; Barile et al., 2009). Applications of whey permeate range from food/feed ingredients to production of industrially related products. These products include lactic acid, vitamin and plastic material polylactic (Aeschlimann and Vonstockar, 1989; Barile et al., 2009; Gbassi et al., 2009; Hugenschmidt et al., 2010). In this part, we will focus on the application of whey permeate as feed ingredients and as media to grow lactic acid bacteria.

4.3 Whey permeate as cultivation media for probiotic

The abundance of lactose allows industrial biotechnologist to utilise whey permeate as ingredients to produce biomass and its metabolites. Most biomass production involves cultivation of lactic acid bacteria (Schepers et al., 2002; Mondragon-Parada et al., 2006; guirre-Ezkauriatza et al., 2010). The encouraging observations have inspired the utilization of whey permeate as a medium to screen lactic acid bacteria isolates for new probiotic strains (Paper II).

As expected, the main metabolite from growing bacterial cells in whey permeate is lactic acid. Between early 1990 to early 2000, the diversity of lactic acid applications increased and inspired efforts to improve production efficiency (Aeschlimann and Vonstockar, 1991).

Supplementing whey permeate with yeast extract allows production of folate and vitamin B12 by selected lactic acid bacteria and propionic bacteria (Hugenschmidt et al., 2010).

4.4 Whey permeate as feed additive

The motivation of including whey permeate as feed additive, especially in piglets, is due to the abundant lactose content. For more than 10 years, lactose has been added into the diet of post weaned piglets and resulted in an improved growth performance and greater numbers of lactobacilli (Mahan et al., 2004; Cromwell et al., 2008). Characteristically, whey permeate retains the sweet taste of milk despite a lack of creamy note, hence desired palatability when added into basal diet. In piglets, lactose is metabolized by β-galactosidase and β-glucosidase (Figure 2). Both enzymes are better known as lactase. These enzymes are produced in the mucosa layer in the small intestine as one of the brush border enzymes (Manners and Stevens, 1972).

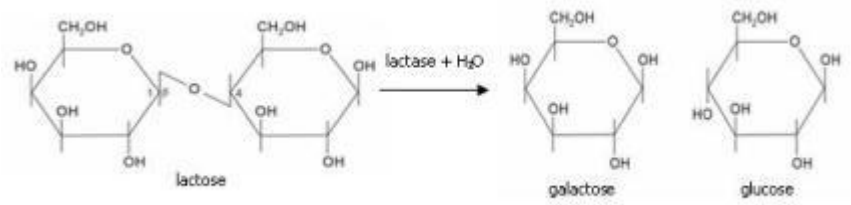

Figure 2. Hydrolysis of lactose into galactose and glucose catalysed by lactase

It has been suggested that piglets benefited from lactose feeding by better feed digestibility and improved intestinal environment. Immediately after weaning, lactose provided simple carbon source for the young digestive tract. At the same time lactose also supports the growth of *Lactobacilli* and helps in keeping a healthy intestinal environment (Mahan, 1992; Cromwell et al., 2008). Increasing the amount of whey permeate does not always translate to improved growth performance. Healthy piglets fed with different whey permeate levels for 35 d did not grow faster compared to the control group. However, over a period of 4 weeks post weaning, piglets exhibited better efficiency in converting diet into daily weight gains. (Paper I). This may be because of piglets age, other brush border enzymes such as sucrase and maltase production increase, which allow young pigs to diversify their source of energy intake (Manners and Stevens, 1972).

The amount of lactose in sow milk is similar to the concentration of lactose in bovine milk. Piglets weaned less than 20 days old are on the advantage due to primed lactase activities carried over from the lactation period. This could be how whey permeate provide more

growth promoting effect in piglets weaned at less than 20 days old then piglets weaned at later age (Pierce et al., 2007). On the other hand, piglets weaned at more than 20 days old are more competitive to combine whey permeate with other carbohydrate sources in feed (wheat, barley, potato) as an energy source. The diversified brush border enzyme may help in improving the efficiency of converting feed into growth (Paper I).

Whey permeate additions consistently improved growth performance parameters. This include average daily gains among piglets weaned at 20 days (Pierce et al., 2005; Pierce et al., 2006) and ratio in converting feed into daily gain among piglets weaned at 28 days (Paper I). Furthermore, it increased the counts of beneficial *Lactobacillus* in pigs weaned at 21 days (Kim et al., 2010). These observations provide argument to include whey permeate, especially for its lactose content, as an alternative to AGP, in reducing the propensity of weaned piglets to develop PWD.

5. Probiotics for pigs

For piglets, a probiotic is expected to provide at least one of the following benefits: (1) stimulating the development of a healthy microbiota, predominated by beneficial bacteria, (2) preventing enteric pathogens from colonisation, (3) increasing digestive capacity and lowering the pH in the GIT, (4) improving mucosal immunity, and (5) enhancing gut tissue maturation and integrity (de Lange et al., 2010). Such high expectations never been directed to any other bacterial group (Mills et al., 2011). Appropriately, a thorough screening process prior to addition to pigs is required.

5.1 Screening

Customized searches for potential probiotic directed for applications in pigs are ongoing. However, the screening process generally maintains the recommendation from FAO/WHO, 2002 which was originally drafted for human applications. Desirable characteristics for a probiotic are recently reviewed (Gaggia et al., 2010) including (1) Non-toxic and non-pathogenic, (2) accurate taxonomic identification, (3) normal inhabitant of the targeted species, (3) survival, colonization and being metabolically active in the targeted site, including resistance to gastric acid and bile, survival in the GIT, and ability to adhere to the epithelium or mucus layer, (4) modulation of immune response, (5) ability to exhibit at least one scientifically supported health benefit to the host, (6) genetic stability, (7) high viability and stability of characteristics throughout food processing, storage and delivery (8) contribute to desirable organoleptic for the finished food products. The stages of evaluating potential

probiotics for human applications span over (1) strain genetic identification, (2) *in vitro* functional characterizations and safety followed by *in vivo* characterizations and safety tests in animals; finally (3) three phases of human trials to determine its safety, efficacy, and efficiency (FAO/WHO, 2001).

Up to the time when this thesis was written, different natural sources of isolations for potential probiotics for pigs have been recorded. These include the pig's GIT tract, feces, sow's milk, fermented feed or other fermented food products (Jacobsen et al., 1999; Chang et al., 2001; Jadamus et al., 2001; Casey et al., 2004; Konstantinov et al., 2006; Collado and Sanz, 2007; De Angelis et al., 2007; Guerra et al., 2007; Kim et al., 2007; Jurado et al., 2009; Martin et al., 2009; Guo et al., 2010; Lahteinen et al., 2010).

Worth mentioning that while in human, strains belonging to the genus *Lactobacillus* or *Bifidobacterium* are the most common, in animal nutrition, strains of *Enterococcus faecium*, or spore preparations of strains belonging to the genus *Bacillus* is currently the most common. These bacteria originated from soil, unlike human probiotics which are mostly isolated from human GIT or food products. However, Bacillus, as vegetative cells or as spores repeatedly showed some efficacy as probiotics in pig trials (Jadamus et al., 2005; Taras et al., 2006; Taras et al., 2007; Simon. 2010).

Phenotypic, metabolic and genetic characterizations on potential probiotic isolates obtained from these various natural sources is essential to historically determine the safety status (Gaggia et al., 2010). European Food Safety Authority (EFSA) introduced a list of microorganisms used as food or feed additives and belonging to Qualified Presumptive as Safe list (QPS) (EFSA Journal, 2007). The list was recently updated with more detailed descriptions on different microorganism groups including new information on *Enterococcus* (EFSA Journal, 2011). In the USA, utilization of microorganism for animal consumption are not specifically regulated, but for livestock production, the path of microorganisms used as a food additive should posses "GRAS" status (Generally Regarded as Safe) regulated by the Food and Drug Administration.

The order by which the screening process proceeding is driven by the final applications. However, in general, the *in vitro* evaluations follow FAO/WHO recommendations quite closely. When the applications in pigs are directed in finding alternative to antibiotics to fight pathogenic infections, one of the first screening steps was to determine whether the isolated strain is pathogenic or not and further its ability to inhibit selected pathogens, which

commonly included different serovar of *E. coli* and *Salmonella* Typhimurium(Casey et al., 2004; Missotten et al., 2009; Lahteinen et al., 2010).

The evaluations on survivability of potential strains through out feed processing and throughout the GIT passage *in vitro* received equal importance (Casey et al., 2004; De Angelis et al., 2007; Lahteinen et al., 2010). Different approaches ranging from applying pelleting treatment (De Angelis et al., 2006), acidified media and media supplemented with porcine or oxgall bile acids (Casey et al., 2004; Lahteinen et al., 2010) to ileum model and *ex vivo* evaluations (Blake et al., 2003; Iyer et al., 2005) were applied. The evaluations follow the rationale that to be able to exert health benefits, probiotics need to arrive at the targeted location (ileum or colon of pigs) alive. Hence the ability to survive feed pelleting process, extreme low gastric pH, and intestinal bile (Blake et al., 2003).

Reaching the ileum of pig, potential probiotics needs to be able to adhere and together with the commensal microbial community, co-colonize the mucosa layer of the pig intestinal epithelium. *In vitro* assessment of these characteristics involve pig or piglet originated intestinal epithelial cell lines (Skjolaas et al., 2007; Lahteinen et al., 2010; Marcinakova et al., 2010). In addition to evaluate adherence to epithelial cell, Intestinal Porcine Epithelial Cell (IPEC) 1 was used to measure the protective potential of *L. sorbrious* to membrane barrier damage caused by *E.coli* F4 infection and the regulation of pro-inflammatory cytokines (Roselli et al., 2007).

Similarly to the human applications, the efficacy of potential probiotics can only be validated in pigs as the host. As most of the screening guidelines were adapted from human probiotic criteria, there might be some discrepancies of how these isolates provide health benefit in pigs.

5.2 Mode of actions – human and pigs

Three modes of action were summarized recently (Kenny et al., 2011; Bron et al., 2012) by which probiotics help in improving human or mammalian health in general. First, probiotics may promote competitive exclusion of pathogens, either by direct inhibitory provided by inhibition activities produced by probiotics, or indirectly through influencing the commensal microbiota. Second, probiotics may enhance epithelial barrier function by modulating signaling pathways that lead to enhanced mucus or defensin production, or by preventing apoptosis or increasing tight junction. Third, probiotic may modulate the immune system of

the host, especially in the small intestine. This region contains a large proportion of the immune modulator capacity of the body, and the population size of the endogenous microbiota for this site is relatively small, allowing transient dominance of ingested microorganisms, which is in this case, probiotics. Figure 3 demonstrates the proposed mechanisms of how probiotic promote gut health in human.

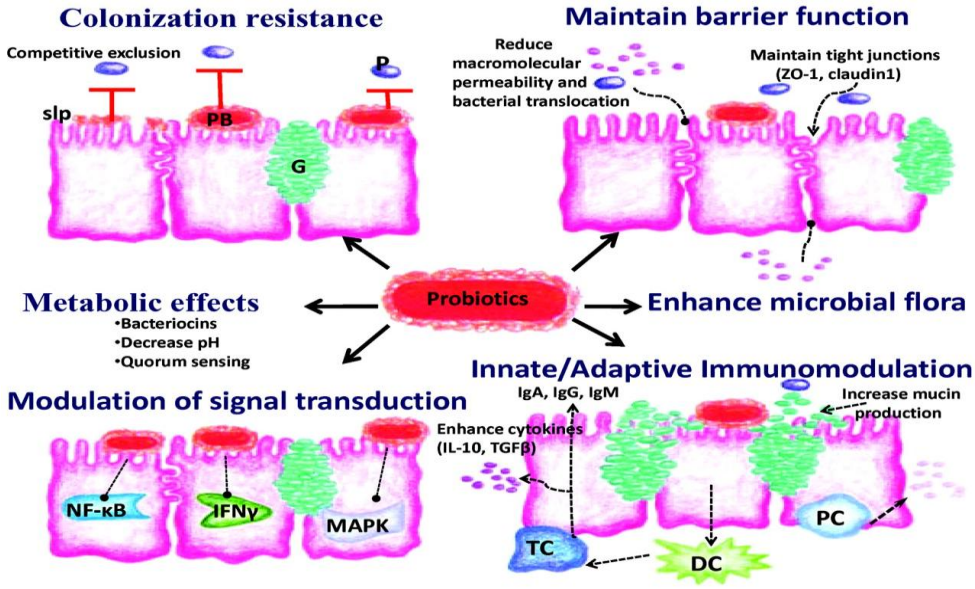

Figure 3. Probiotics stimulates gut health in human (Sherman et al., 2009)

Specifically for application in pigs, the benefits observed from administrating probiotics have been mostly related to changes in growth performances, diarrhoea symptoms, changes in the gastrointestinal morphology, and changes in selected microbial communities (Casey et al., 2007; Collado and Sanz, 2007; Konstantinov et al., 2008). An additional benefit for pigs is the improved availability of feedstuff upon administration of probiotics (Kenny et al., 2011). The mechanisms of how probiotic influence pig physiology is not easy to summarize. The challenges come from different regimes that have been applied in pig trials which include different probiotic strains, duration of administrations, dose of viable organisms and the life cycle stage of the pigs. An added variable could also be the pigs genetic variant (Bomba et al., 2002; Scharek et al., 2007; Konstantinov et al., 2008; Pieper et al., 2008; Schierack et al., 2009; Kenny et al., 2011).

Affected dietary absorptions which support the growth of pigs after receiving probiotics were observed in some trials (Lodemann et al., 2006; Konstantinov et al., 2008; Lodemann et al., 2008). Increased L-glutamine transport and increased ion secretion was reported in post weaned piglets fed with *Bacillus cereus* or *Enterococcus faecium*. Furthermore, probiotics

provides additional sources for dietary enzymes such as lipase, amylase, phytase and protease {{1249 Kim,Eun-Young 2007}}.

Unlike in human, studies on protective activity of probiotics as membrane barrier in pigs are rare (Kenny et al., 2011). However, few important observations from *ex-vivo* studies may help to understand the mechanism. The protection against membrane barrier disruption by pathogen appeared to be multi factorial, including induction of mucus secretion from goblet cells (Caballero-Franco et al., 2007), maintaining membrane integrity by IL-10 regulation and maintenance of the tight cell junctions between cells (Roselli et al., 2007). The mechanism of maintaining tight cell junctions and membrane integrity is especially relevant to post weaned piglets. As compromised intestinal barrier function which increase the risk of pathogen infection are common during the weaning period (Wijtten et al., 2011).

Probably most reviewed is how probiotics modulate the immune response in the intestine. Probiotics may produce defense mechanism to the cells through induction of anti-inflammatory cytokines, and repression of pro-inflammatory cytokines, from enterocytes and intestinal immune cells which were directed to the sites of inflammation by probiotics (Walsh et al., 2008; Wang et al., 2009). The toll-like receptors (TLR) are regarded as one of the gut's primary means of detecting and initiating responses to microbial molecular markers. Studies in pigs recorded that *B. animalis* feeding affected the expressions of TLR-2 lymph nodes when fructo-oligosaccharides were included in the diet (Trevisi et al., 2008). Furthermore, the expression of tumor necrosis factor-α was positively correlated with TLR-2 and negatively correlated with the amount of *B. animalis* DNA. The activation of the immune system was recorded in *S. enteric* Typhimurium-challenged piglets. Administration of *E. faecium* NCIMB 10415 resulted in lower CD8+ intraepithelial lymphocytes (Szabo et al., 2009).

5.3 Probiotics in pig trials

The growing although yet conclusive knowledge about the probiotic mode of actions for human applications, derived from *in vivo* studies in pigs, might have inspired animal scientists. The idea of adding living organisms to modulate the gut microbiota in pigs, which previously have been considered to be similar to human, seems logical as an alternative to using antibiotics. There have been growing numbers of research activities in the past 10 years to elucidate probiotics applications in pork production, especially relating to prevent or treat early life gastrointestinal diseases (Roselli et al., 2005; Jurado et al., 2009; Modesto et al., 2009). Some of these trials included strains which were previously isolated for human

applications: *L. rhamonsus* GG, *L. paracasei*, *L. casei, L. plantarum L. pentosus*, *Bifidobacteria animalis, Streptococcus thermophilus* (Siggers et al., 2008; Cilieborg et al., 2011; Trevisi et al., 2011).

The challenges and questions of understanding how probiotic works in pigs are as great as or even greater than they are in human applications. Most of the proposed mechanisms by which probiotic administration benefits the host are related to maintaining a healthy balance of gut microbiota. A balanced gut microbiota has been linked to the well-being of the host. Some of the critical roles of the gut microbiota in human include vitamin and co-factors productions, metabolism of otherwise indigestible nutrients, detoxification, covering the gut surface to physically reduce the possibility of pathogen attacks, production of antimicrobial, maintenance of gut barrier function and promotion of anti-inflammatory responses (Kenny et al., 2011). Gut microbiota plays an important role to "prime" the neonatal immune system which in turn is able to perform adult functional system for recognising pathogens and for dealing with new food antigens (Bailey et al., 2005).

In animal nutrition, probiotic bears a definition of viable microorganisms, which lead - after sufficient oral intake - to beneficial effects for the host animal as exhibited by an improvement of the intestinal microbial balance (Fuller and Gibson, 1997). This places similar emphasis to the application of probiotic for human (Reid et al., 2006; Reid. 2008). Widely accepted claims of how probiotics benefit animals include the improvement of growth performance (daily weight gain, feed intake, and feed conversion ratio). However, the mechanisms for these observations are still elusive. There have not been specific guidelines to evaluate probiotic attributes for animal applications. Therefore, the guidelines for human applications are still considered also relevant for animal usages (Simon. 2010; Kenny et al., 2011).

Simon O (2010) argued that for the applications of probiotic in animals, there needs to be a different approach from the human applications which related to the administration of the probiotics. The survival rate of bacterial cells which are incorporated into feed prior to pelleting needs to be considered. Furthermore, the questions of the points and duration of probiotic of administration are considered crucial.

5.3.1 Timing

It is well understood that in their life cycle, pigs encountered critical periods which are immediately after birth and two week post-weaning (Kenny et al., 2011). During early life, colonization patterns varied greatly on the basis of genetic relatedness and environmental influences. Initial colonization of the gut ecosystem is crucial as it helps in 'programming' the expression of genes which are desirable to fight against environmental pathogens (Siggers et al., 2008). It is also postulated that ideally, neonates should pick up a microbiota at birth which would improve nutrient quality by providing vitamins, amino acids and short chain fatty acids (Petri et al., 2010). Hence, addition of probiotic to neonates provide early intervention to yet stabilized intestine microbiota as beneficial community helps to educate the 'naive' host immune system which indirectly help to fight against environmental pathogen invasions (Bailey et al., 2005; Stokes et al., 2004)

A different argument proposed that immunity provided by probiotic administration to the sow is carried over to the litter hence providing early protection even before birth (Simon. 2010). Feeding of *B. subtilis cereus* var *toyoi* NCIMB 40112 to sows early in pregnancy resulted in higher IgA in the feces from the sows and later decreased amount of IgG in the jejunal content of piglets (Scharek et al., 2007). Moreover, administration of *Enterococcus faecium* NCIMB 10415 to the sow resulted in reduced transfer of Chlamydia from infected sows to piglets (Pollmann et al., 2005).

Immediately after weaning, piglets go through multidimensional changes in their gut physiology as described in a recent review (Lalles et al., 2007b). The GIT-related disorders in postweaned pigs is not only the consequences of changes in GIT morphology and function, but also from drastic changes in the enteric microbiota and immune system (Konstantinov et al., 2004; Bailey et al., 2005).

Influencing the gut microbiota by adding probiotic in the diet is hypothesised to help piglets improve nutrient digestibility, local and systemic immune system and in whole health (Lalles et al., 2007b; Trevisi et al., 2007; Bosi and Trevisi, 2010). However, thus far, the results have been mixed with most probiotic administration that did not result in significant improvement in measured parameters (Taras et al., 2006; Simon. 2010) or even caused a decrease in piglet condition (Trevisi et al., 2011).

5.3.2 The right agent, amount, duration

Pig trials involving probiotic bacteria feeding commonly apply strains belonging to groups lactobacilli, spore forming *Bacillus* or enterococci (Bosi and Trevisi, 2010; Gaggia et al., 2010; Simon. 2010). Other less frequently used include the non pathogen *E. coli* (Schroeder et al., 2006), yeast isolate such as *Saccharomyces cerevisiae* (Mathew et al., 1998), or *Bifidobacterium* (Shu et al., 2001). It is difficult to perform metanalysis as most of the responses are strain specific. In most cases, the choice of including a certain probiotic strain motivated by promising probiotic traits from in vitro assessments (Paper I, Casey et al., 2007; Konstantinov et al., 2008). In addition, the variations also are coming from the farm or conditions in experimental station and the genotype and immune system and microbiota in the piglets being studied (Kenny et al., 2011).

The period of probiotic administration may also needs to be reconsidered. Time duration of applying probiotic in pigs trial varies greatly. Some studies added probiotics for as long as 50 days started from gestating sows to first weeks of postweaning periods to as short as 3 days in neonate piglet trials (Scharek et al., 2005; Scharek et al., 2007; Schierack et al., 2009).

The amount of probiotic added into pigs is also important. In general, similar number of viable cells, in the range of 10^8 to 10^{10} CFU per day are added in pig trials (Taras et al., 2007; Konstantinov et al., 2008). However, the age of the piglets being administrated with probiotic need to be taken into account. Addition of probiotics in abundance is understood as additional antigen by the host. Hence, immature immune system may not be capable of responding which may generate unnecessary inflammation and worsen diarrhoea.

The initial status of experimental piglets which reportedly affect the efficacy of probiotic. Probiotics are fed to previously infected piglets (Amezcua et al., 2007; Casey et al., 2007; Konstantinov et al., 2008; Daudelin et al., 2011) or to healthy animals (Scharek et al., 2005; Lodemann et al., 2006; Schroeder et al., 2006; Zeyner and Boldt, 2006; Canibe et al., 2007; Guerra et al., 2007; Scharek et al., 2007; Schierack et al., 2007; Takahashi et al., 2007; Bernardez et al., 2008; Solano-Aguilar et al., 2008). The parameters in these studies included growth performances, incidence of diarrhea, amount of pathogen shedding days, and production of localized immune responses (IgG or IgA).

5.3.3 Infection model vs healthy piglets

The effects of subclinical infections with pathogens are likely to be important with respect to production parameters as energy spent fighting detrimental bacteria is energy lost to the animal, and farmer, in terms of growth and efficient feed conversion. It is among these compromised, but not overly ill animals probiotics may be the most helpful (Kenny et al., 2011). Precisely, one criticism at challenged models to induce PWD is that the incidence and severity of the diarrhoea observed is often less than that experienced in commercial herds where dietary anti-microbial compounds are not introduced. Indeed, it has always been a challenge to draw the fine line in using an *E.coli*-challenge model between causing a mild level of diarrhoea and unintentionally causing enterotoxaemia and mortality during the experiment (de Lange et al., 2010). As an example, (Bosi and Trevisi, 2010) observed that *Salmonella enterica* Typhimurium infected piglets fed with *Bifidobacterium animalis* suffered from reduced amount of IgA. The authors argued that when young animals are infected heavily, the reduced amount of IgA-secreting cells will not be rapid enough to protect the invasion (Bosi and Trevisi, 2010). Another challenge is to include non-infected group in a challenged study. Cross-contamination from the infected animals into the non-infected group may be more common than being revealed (Paper III).

Resistance by farm technicians due to reduced efficacy in the farm level as suggested (Bosi and Trevisi, 2010) possibly resulted from (1) competition by already established commensal microbes in the pigs; (2) poor delivery method which resulted in insufficient viable probiotics that reach pigs' GIT system; (3) ageing of probiotic prior to being consumed; (4) variety of intestinal fermentation from one pig to another, which may be related to different diets; (5) probiotic may replaced favorable commensal colonies.

6. Plant extracts as feed additive for pigs

Herbs and spice extracts have been used extensively in different parts of the world to treat gastrointestinal diseases (Hill et al., 2006; Burns et al., 2010; Lam et al., 2010). In addition to being inhibitory against enteric pathogens (Xia et al., 2011), studies suggest that Traditional Chinese Medicine modulate gut microbiota of hyperlipidemia (Zhang, 2003). In production animals, empirical evidence proposes that plant extracts may offer benefits in boosting the immune system (Wenk. 2003). Furthermore, improved growth performance is observed (Ding et al., 2011).

The challenge to elucidate using plant extract as alternative to manage PWD is the limited understanding of bioactive compounds. Most studies have used mixture of compounds which do not allow the investigation of the efficacy of each component (Gallois et al., 2009). *Fructus mume* extracts have been included in medicinal drinks in China (Xia et al., 2011). Mixed with more than 3 other extracts, *Fructus mume* help in reducing viral infections in chickens (Cheng He, personal communications).

Extract of *Fructus mume* exhibit inhibition towards *Escherichia* (Sakagami. 2001)*coli* {{834 Sakagami, Yoshikazu 2001}} (Paper IV). Furthermore, when tested on porcine jejunal intestinal epithelial cell line, extract of *Fructus mume* reduced the expression of pro-inflammatory cytokine, IL-18 (Paper IV). Providing an *in vitro* challenge of the intestinal epithelial cell line with *E. coli*, it is possible to further evaluate whether delayed expression of IL-18 is maintained which may help in regulating innate immune response during weaning.

Table 1.

Pig trials using probiotics

	Pig race	Host	Weaning age (d)	Start of treatment (d)	Probiotic strain(s)	Amount (CFU/kg feed)	Delivery	Duration (d)	Challenge	Time of challenge (d)	Observed results	Reference	Year
1	Landrace x Duroc	Sow Piglets	N/A	-90 14	*Bacillus cereus* var. *toyoi*	3.3×10^8 1.4×10^9	Mixed with feed and pelleted	118 14 - 56	No No	N/A N/A	longer nursing days in probiotic group reduced incidence of liquid feces during weaning period improved feed conversion rate at week in weaning period loss of weight up to 35 d	Taras et al.	2005
2	Landrace x Duroc	Sow Piglets	N/A	- 90 14	*Enterococcus faecium*	1.6×10^9 2×10^8	Mixed with feed	- 90 to + 28 14 - 56	No	N/A	$CD8^+$ decrease at 14 d Decreased IgG at 56 d	Scharek	2005
3	Landrace x Duroc	Sow Piglets	N/A	- 90 14	*Enterobacter faecium*	1.4×10^9 2.0×10^8	Mixed with feed and pelleted	118 14 - 56	No No	N/A	No significant improvement in growth performance or incidence of diarrhea reduced incidence of liquid feces during weaning period	Taras et al.	2006
4	Landrace x Large-White	Piglets	21	21	*L. sorbrius*	1×10^{10}/ml	Orally added	14	*E. coli* F4	28	increased days of diarrhoea reduced counts of *E. coli* F4 improved ADG	Konstantinov et al.	2008
5	Costwold	Piglets	17	17	*B. subtilis*	N/A	Mixed with feed	14	*E. coli* K88		Decrease diarrheal faeces at 24 h post infection Higher Bacteriodetes	Bhandari et al.	2008

	Pig race	Host	Weaning age (d)	Start of treatment (d)	Probiotic strain(s)	Amount (CFU/kg feed)	Delivery	Duration (d)	Challenge	Time of challenge (d)	Observed results	Reference	Year
6	Costwold	Piglets	17	17	*E. coli* UM-2 and UM-7	8×10^{10}	Mixed with feed	17	*E. coli* K88	24	Increased ADG Reduced diarrhoea Increased microbial diversity	Krause et al.	2010
7	Duroc x Landrace x Large-White	Piglets	28	28	*S. cerevisiae* ssp. *boulardii* *P. acidilactici*	2×10^{9} 1×10^{9}	Mixed with feed	4 weeks 3 weeks	No No	N/A	improved feed conversion rate less counts of faecal *E. coli*	Le Bon et al.	2010
8	Yorkshire Landrace	Sow Piglets	N/A 21	-28 1	*P. acidilactici (PA)* *S. cervisiae boulardii (SC)*	2.5-3.5 x 10^{9} 1×10^{9}	Mixed with feed Direct tube feeding	-28 to +21 29	*E. coli* F4	28	PA & SC groups: reduced *E. coli* F4 attachments to ileum mucosa PA & PA+SC groups: increased IL-6 in ileum	Daudelin et al.	2011
9	Landrace x Large-White	Piglets	21	21	*L. rhamnosus* GG	6×10^{9} / day	Mixed with feed	14	*E. coli* F4	7	Reduced ADG Decreased villus height	Trevisi *et al.*	2011
10		Piglets	28	28	*L. plantarum* *W. viridescens*	1×10^{10}	Mixed with feed	11	*E. coli* F4	2	Decreased in days with diarrhoea Improved amount of butyric acid Change in Firmicutes in colon	Manurung *et al.*	2013 (in prep)

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Whey permeate improved the feed conversion ratio of post weaned piglets, without disturbing the intestinal environment

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Abstract

Previous studies that utilized whey permeate or lactose as feed additives to post weaned piglets have been inconclusive about the dose and benefical effects to improve growth perfomance and gut health. An experiment was performed, in which whey permeate powde was added (0, 60, 120, 180, 240 g/kg as fed) to evaluate the effects on growth performance and the intestinal health in weaning piglets. In total, 100 piglets were weaned at 28 days of age blocked on the basis of litter, and placed in pen two by two. Each pen was given one of the 5 dietary treatments providing 10 replicates per treatment. Piglets were fed *ad libitum* for 35 days post weaning. One of the piglets from each pen was sacrificed at Day 14 for determinations of short chain fatty acids, lactic acid, and microbiological contents of the different gastrointestinal tract parts and for analyses of colonic microbiota. Microbiota communities were determined using Illumina Hiseq platform by sequencing V1 hypervariable region of the 16S rRNA gene amplicons. Different levels of whey permeate additions did not affect significantly the average daily gain nor average daily feed intake. The ratio of feed conversion to weight gain improved starting from the later phase after weaning, until the end of the experiment (14 – 35 days). The relative abundance of phyla was stable within the first 2 weeks after weaning except for a decrease in Bacteroides at 60 g/kg, mostly due to reduction of *Prevotella*. The study revealed that piglets weaned at 28 days exhibited the ability to metabolise feed-in whey permeate up to 240 g/kg without giving any intestinal problems.

Implications

The economical aspect of obtaining better yield and at the same time maintaining the health of pigs immediately after weaning is of great interest. The application of whey permeate to address the challenge needs to be better understood. Particularly, variability of whey permeate produced as a co-product from cheese industry may affect the response of piglets metabolically resulted in a compromised growth performance and gut health. This study showed that feeding a commercial whey permeate powder (Variolac® 830) as low as 60 g/kg improved feed conversion ratio and did not affect the balance of colonic microbiota.

Introduction

Environmental, social and diet changes during weaning process often cause the sub-optimal growth performances and diarrhoea in piglets. Reductions of feed intake, infections, changes

in gut morphology and enteric microbiota have been linked to weaning problems (Lalles et al. 2007, McCracken et al. 1999, Wijtten et al. 2011). Adding fermentable carbohydrate, including lactose, has been considered as viable approach to eleviate detrimental effects of weaning (Pierce et al. 2005,Pierce et al. 2006).

For the last 10 years, lactose has been added into the diet of post weaned piglets and resulted in the improved growth performance and greater numbers of lactobacilli (Cromwell et al. 2008, Mahan et al. 2004). The disaccharide, lactose is metabolized by the intestinal mucosa β-galactosidase and β-glucosidase. Both enzymes are better known as lactase (MANNERS and STEVENS. 1972). Lactase activity of piglets is highest in the proximal site of small intestine especially among the neonates. The activity starts to decrease during the first 2 months of life while activities of other disaccharides such as sucrase and maltase increase (MANNERS and STEVENS. 1972).

Reports about studies on the effect of feeding lactose to post weaned piglets mainly focus on the improvement of growth performances, changes in the gut morphology and few selected bacterial counts (Cromwell et al. 2008,Molino et al. 2011). However, it is not confirmed at which level these changes happens and how high level of lactose are necessary for different ages of piglets after weaning.

Lactose has been reported to improve the diversity of attached lactobacilli in the intestine of post weaned piglet (Krause et al. 1995). However, this study was limited to culturable bacteria. Gut microbiome changes due to lactose feeding is still not well studied. Denatured Gel Gradient Electrophoresis (DGGE) on DNA obtained from digesta of piglets proximal colon show that Eubacteria and *Lactobacillus* richness (Shannon-weaver indices) is not affected by lactose feeding up to 12 % lactose did not change (Molino et al. 2011).

Whey permeate, a byproduct from the cheese industry, is an economical source of lactose (up to 830 g/g). Abundant and bulky, whey permeate application and valorisation is of great importance to the sustainability of dairy processing industries (Barile et al. 2009,Smithers. 2008). Characteristically, whey permeate retains the sweet taste of milk despite a lack of creamy note, hence desired palatibility when added into basal diet.

This study was performed to determine the appropriate level of whey permeate (WP) feeding to support a potentially increased growth of post weaned piglets and to determine whether high amount of whey permeate feeding alters colonic microbial communities investigated by next generation sequencing.

Materials and Methods

The animal experiment was conducted at Aarhus University, Department of Animal Science, Denmark. The procedure was approved by the Danish Animal Experiments Inspectorate.

Animals and housing

A total of 100 crosbred piglets (Danish Landrace x Yorkshire x Duroc) from 10 litters in Aarhus University Swine Herd, Foulum, Denmark were involved in the study. Ten piglets from each litter were weaned at 28 ± 1 day and a body weight (BW) of 7.84 ± 0.05 kg prior to being transported to pens (184 x 82 cm, of which 82 x 82 was slatted) two by two. Each pair was alloted to one of the five treatments. No physical contact between piglets from different pens was allowed.

Diets and Feeding

Piglets were fed with dry basal diet (Table 1) with different levels of in-feed whey permeate (Variolac® 830, Arla, Denmark). The 5 treatment groups were: WP0 (no whey permeate), WP60, WP120, WP180 and WP240 (60; 120; 180; and 240 g/kg whey permeate additions, repectively). Inclusion of whey permeate was compensated with wheat level in the experimental diets. The amount of dehuled toasted soybean meal and the addition of the synthetic amino acids lysine, methionine, threonine and tryptophan were adjusted to optimize the diet with regards to protein and amino acid composition (Table 1). The animals were fed the experimental diets *ad libitum* throughout the study and given free access to water. Feed uptake and weight of the pigs were registered weekly.

Experimental procedure

On Day 14 at BW of 11.9 kg ± 0.18, one pig from each pen was sacrificed 3 h after morning meal. The remaining piglets were kept in the pens to study the effect of experimental diets on growth performance during the first 5 weeks post weaning. The temperature of the nursery was maintained at 28 ºC.

The pigs were sacrificed by a captive bolt gun. The gastrointestinal tract (GIT) was immediately removed, measured and divided into 8 segments: stomach, 3 equal (length) parts of the small intestines, caecum, and 3 equal (length) parts of the colon including the rectum. The total contents of each segment were weighed and within 5 min, the pH was determined. Digesta from the stomach, the distal segment of the small intestines, the caecum, and the spiral colon (from here on will be written as colon) were immediately analysed for microbial contents. Residual digesta samples from 8 segments of the GIT were stored at -20 ºC for

analyses of dry matter (DM) and short chain fatty acid (SCFA), and lactic acid concentrations as previously described (Canibe and Jensen. 2007). Dry matter content from the digesta was determined by freeze-drying the samples. To express the results of chemical analyses of the diets in DM percentage, DM was determined by drying the samples at 103 ºC until constant weight was reached (European Union, 1971).

Digesta from colon were collected and stored at -20 ºC until used for DNA extraction. Tissue samples from distal stomach, small intestines, caecum, and colon were stored in 10% neutral buffered formalin for histology and morphometric measurements.

Microbial determinations

Aproximately 10 g digesta samples were transferred rapidly after collection under a flow of CO_2 into a CO_2-flushed plastic bag and diluted 10 times with a pre-reduced salt medium (Holdeman et al., 1977) followed by homogenisation in a stomacher blender (Interscience, St. Nom, France) for 2 min. Then, 10-fold dilutions were prepared in peptone water for the feed samples and in pre-reduced salt medium for the digesta samples by the technique previously described (MILLER and WOLIN. 1974). Samples (100 µl) were plated on non selective and selective media. Total anaerobic bacteria in digesta samples were enumerated by culturing the samples in roll tubes containing pig colon fluid-glucose-cellobiose agar (Holdeman et al., 1977) and incubating anaerobically at 37 ± 1 ºC for 7 days. Lactic acid bacteria were determined on de Man, Rogosa, and Sharpe (MRS) agar (Merck) after anaerobic incubation at 37 ºC for 2 days for digesta samples, respectively. Enterobacteriaceae including coliforms in digesta samples were enumerated on McConkey agar (Merck) after aerobic incubation at 37 ± 1 ºC for 1 day. Yeasts and molds were enumerated on malt chloramphenicol agar (MCA) [10 g/l of glucose (Merck); 3 g/l of malt extract (Merck); 3 g/l of yeast extract (Merck); 5 g/l of Bacto peptone (Merck); 50 mg/l of chloramphenicol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); and 15 g/l of agar (Merck)] following aerobic incubation at 30 ºC for 3 days for feed samples and aerobic incubation at 37 ºC for 2 days for digesta samples.

Histomorphometry

Formalin-fixed tissue samples were processed routinely for histology, embedded in paraffin wax, cut at 3 µm, and stained by hematoxylin and eosin (H&E). Measurement of the total thickness of mucosa (villous height and crypt depth combined) was done under blind conditions as previously described (Marion et al. 2005) with slight modifications. An

overview observation across 5 GI tract sections was performed. Upon the lack of obvious lesions, including athropy in these samples, 20 random determinations of total thickness of mucosa were performed for jejunum and colon samples from all pigs. Measurement of the total thickness of mucosa (villous height and crypt depth combined in jejunum) was done under blind conditions as previously described (Marion et al. 2005) with slight modifications. Measurements were done using a Zeiss Imager M1 microscope coupled with Axio camera to the PC computer supported by the AxioVision rel 4.8 software (Carl Zeiss GmbH, Germany).

Isolation of DNA

Total DNA representing the colonic microbiome was extracted from individual colonic digesta samples using the QIASymphony virus/bacteria mini kit (Qiagen, Mainz, Germany) according to the manufacturer's instructions. Samples preparations were conducted as follow: digesta samples were prepared as a 10% (w/w) solution in PBS. Samples were beaten using metal beads at 15 Hz twice for 1 min interval with 15 sec pause in between. Purity of the extracted DNA was determined using UV absorption spectrums including OD 260/280 ratio on a Nanophotometer (Implen, Munich, Germany).

Polyemerase chain reaction amplicon construction and sequencing

The following PCR primers that flanked the V1 hypervariable region of bacterial 16S rRNAs Bact 64f (5'- CYTAAYRCATGCAAG-3') and Bact 109r (5'-CACGYGTTACKCA-3') (Yu et al. 2006) were used. Unique DNA sequence identifiers (barcodes), which allowed us to pool samples together and subsequently to segregate the sequence reads for each sample, were attached to the 5' ends of forward and reverse primers. The barcodes were designed to be 6 bp with at least one base difference from one another. A list of total 50 barcodes in this study was included in Supplementary Table S1. The barcoded-primers were designed and purchased from DNA Technology, Aarhus, Denmark. PCR mixes contained final concentations of 1X polymerization buffer, 0.1 mM concentrations of each deoxynucleoside triphosphate (dNTP), 0.2 µM of each of both forward and reverse barcoded-primers and 4 U of Taq DNA polymerase (Applied Biosystem, Denmark). To each reaction 4 µl of the extracted template-DNA was added. The reaction mixtures were subjected to initial denaturation cycle at 94 ºC for 3 min, followed by 40 cycles at 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 30 s, and an extension step at 72 ºC for 5 min. A negative control containing only PCR mix and buffer was included in the PCR run.

The quality of the product was evaluated in a Bioanalyzer 2100 (Agilent, CA, USA) using DNA 1000 LabChip (Agilent, CA, USA). Only PCR products without contaminant bands were used for sequencing. The PCR products from different pigs were pooled in equimolar ratios based on Nanophotometer (Implen, Munich, Germany) readings prior to phenol/chloroform precipitations. Products were eluted in EB buffer and submitted for sequencing, including base calling, at the University of Copenhagen Sequencing Center, Denmark. Sequence data processing was performed using an open source Linux based software (ftp://genomics.dk/pub/BION; Larsen et al. *in prep*) . The steps of sequencing data processing was described in Supplementary Figure S1.

Statistical analysis

A mixed model was used to perform statistical analyses to estimate the effect of diet and segment along the GI-tract on various response variables in digest (Canibe et al. 2008). Diet, segment of the GI-tract and the interactions between diet and segment, were considered as fixed effects. To capture the correlation between measurements in different segments of the GI-tract on each pig, the random errors were allowed to be correlated (the statement 'repeated' in SAS). The analyses were performed with SAS for Windows ver. 8.2 (SAS Institute, Cary, NC, USA). When there was an overall effect of diet at an alpha of $P \leq 0.05$, differences between means were compared pairwise using an *t*-test.

The statistical analyses to determine the effect of diet on total thickness of mucosa and on colonic microbiota were performed using a one-way ANOVA and a non-parametric one way ANOVA (Kruskal-Wallis) test, respectively. Each piglet was determined as experimental unit. When there was an overall effect of diet at $P \leq 0.05$, differences between means were compared pairwise using Dunn t-test.

Results

Feed chemical analysis

Chemical composition of feed is presented in Table 2. In general, the amount of ash increased when there more WP added to feed. However, the amount of amino acids are similar across all experimental diets.

Growth performance and gut morphology

The piglets remained healthy with no clinical symptoms throughout the 35 days of experiment. The effect of dietary treatment on piglet average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio are shown in Table 3. Feeding whey permeate at different concentrations did not affect average daily gains. However, there are numerical increase in body weight during Day 14-35 in WP60 piglets. Average daily feed intake decrease during the same period (P = 0.02) only in the WP120 piglets. Other WP piglets maintained their appetite towards WP additions. Furthermore, WP addition as low as 60 g/kg improved feed intake to growth ratio (P = 0.01).

Phyisco chemical characteristics of digesta from different GIT parts are shown in Table 4. Dry matter contents were not affected by different WP additions. Within the same GIT section, nor was pH affected by diet treatments.

Addition of WP did not alter the morphology of jejunum and colon epithelial structure (Figure 1). No villous atrophy was observed in different GIT parts accross different diet treatments.

Short chain fatty acids and lactic acid

The concentration of lactic acid and volatile fatty acids in various segments of the gastrointestinal tract of the piglets after being fed with different amounts of whey permeate are shown in Table 5. Adding WP at different doses in the feed did not change the concentration of acetic acid, propionic acid butyric acid, and lactic acid. Lactic acid were most abundant in the stomach and depleted throughout transit and become undetected in colon. On the other hand, butyric acid were abundant in caecum and colon but no significant differences were observed among treatments.

Microbiology

The effect of whey permeate on selected microbiological counts are shown in Table 6. Diets affected the amount of lactic acid bacteria. WP240 piglets exhibited significantly less LAB counts compared to other groups in ileum (P = 0.01). There was also a quadratic effect of WP and GIT segments on the Enterobacteriaceae including coliform counts. Feeding WP at 60 or 120 g/kg reduced the coliform counts in caecum and colon (P = 0.002). However, the counts in these sections stayed the same among piglets fed with highest WP (240 g/kg) compared to control group. Furthermore, dietary treatments did not change the number of yeasts or total anaerobic bacteria in the different GI tract parts of piglets.

Microbial community in colon

Illumina HiSeq platform resulted in more than 26 million single reads in total. A total of 9 phyla comprised 99.7% of the community (Figure 2). The abundance of these 9 phyla was relatively stable accross experimental diets except for the phyla Bacteroides and Tenericutes. Within Bacteroides phylum, there was a numerical decrease of *Prevotella* abundance in colonic samples in WP60 pigles relative to all other treatment groups and it was significantly lower against WP240 group (P = 0.04) . Significant decrease in abundance of the order RF39 (P = 0.007) was responsible for the changes in Tenericutes phylum abundant. WP60 piglets showed less RF39 abundance when compared to WP120 piglets. *Clostridium*, a genera belonging to Firmicutes phylum and is known as butyric acid producer, was most abundant in colon. A list of top most abundant 30 phylotypes observed from the Illumina sequencing is provided in Supplementary Table 2. Additions of whey permeate at different levels did not affect diversities of colonic microbiota (Shannon-diversity indices) (Figure 2E).

Discussion

The experiment was conducted to assess the hypothesis that abundant amount of whey permeate (WP) feeding increase the amount of LABs and improves growth performance of post weaned piglets. In this study, lactose was provided in the form of whey permeate powder (Variolac830®, Arlafoods, Denmark) containing 830 g/kg lactose. In none of the treatments, even up to 240 g/kg WP which equals to 200 g/kg lactose as fed, did the amount of LABs increase or changed the colonic microbiota significantly. On the contrary, LAB counts were lower in piglets received 240 g/kg WP. However, there was an improved feed conversion to weight gain ratio starting from the later phase after weaning, until the end of the experiment (Day 14 – 35).

Weaning is frequently associated with a sudden decrease in feed intake, which results in a drastic reduction in growth. Results from the present study exhibited no changes in average daily gains (ADG), average daily feed intakes (ADFI), or feed conversion ratio (G:F) among piglets during the first phase (1 to 14 d). These observations were in contrast with previous results that reported higher ADG and ADFI during initial (week 1-2) post weaning among piglets fed with 25 to 30% lactose (Mahan et al. 2004)or 15 to 25% (Kim et al. 2010). Additionally, there was a linear ADG and ADFI increase during mid- and late-post weaned phases (week 3-4) among piglets fed with 10 % lactose (Cromwell et al. 2008). The contrasting obversations in the present experiment might be caused by the different in weaning ages. In our study, the piglets were weaned at 28 days whereas experimental animals in previous reports were weaned at 21 days (Cromwell et al. 2008, Mahan et al. 2004, Pierce et al. 2007). On this note, it was reported that different weaning ages may be responsible for different responses to lactose addition. The beneficial of lactose declined with the increase in age at weaning (Kim et al. 2010, Mahan et al. 2004).

As the piglets grew older, we observed an improved feed to growth conversion ratios (14 – 35 days period). This observation maybe due to increased activities of maltase and sucrase when the pigs age (Marion et al. 2005) suggesting the adaption of the young intestine to more complex diet. Lactose in whey permeate may affect the growth of piglets more when they are weaned at younger age due to the high activities of lactose. Lactose may no longer be the only or preferred carbon source for piglets weaned at 28 days considering their diversed digestive enzyme activities. This could also explain the decrease in the feed intake (ADFI) and growth to feed ratio (G:F) during the later phase of the experiment (14-35 days) among

WP piglets compared to control groups. Piglets required less amount of feed to exhibit the same ADG, which indicated an efficient conversion of feed being metabolised for gaining energy required to grow.

Whey permeate feeding within the initial post weaned phase (2 weeks) did not affect the morphology of gastrointestinal epithelial surface in our study. Transient small intestinal villus atrophy is a hallmark of "stressed" piglets during weaning which may be alleviated by improved feed intake (Lalles et al. 2007). The normal morphology of jejenum and colon in this study confirmed that all piglets were healthy. Furthermore, no signs of villus athrophy may explained the nonexistence of diarrhoea. Similarly, lactose feeding up to 12 % did not change the intestinal mucosa structure of weaned piglets (Molino et al. 2011). However, inclusion of 15% lactose together with inulin increase the villous height in jejunum when compared to non-inulin lactose consumption. But the effect dissipated when lactose level was increased to 33% (Pierce et al. 2006). The authors suggest that lactose is a less affecting to the small intestine mucosal structure than inulin.

Production of short chain fatty acids (SCFA) in the large intestine relies on the amount and composition of the undigestable nutrients and on the microflora present (Macfarlane and Macfarlane. 2003). In the present study, WP feeding did not affect SCFA concentrations (acetic acid, butyric acid, and propionic acid) in different GT parts. These results did not agree with an increased butyrate production in high (215 g/kg) lactose inclusion (Pierce et al. 2007). Relative to our study, which added whey permeate rather than lactose, a direct comparison may be problematic. Whey permeate contains residual mineral and salts as impurities which may have affected the palatibility of the feed resulting in the numerical decrease of feed intake during the later phase of weaning (14-35 days). SCFA especially butyrate has been proposed as a major energy source for colonocyte (Kien et al. 2000). However, as the brush border enzymes continue to maturize as the pigs grow (Marion et al. 2005), decrease in feed intake did not limit piglets to gain weight.

A combined assesments from culturing digesta samples on selective media and next generation sequencing (Illumina Hiseq), provided a general picture that the microbiota was only slightly affected by diet treatments. A reduction of LAB counts among WP180 and WP240 piglets, especially in caecum, was one of them. Similarly, at 12.5%, lactose reduced the number of faecal *Lactobacilli* populations (Pierce et al. 2007) but in a different study, at 25%, lactose improved faecal *Lactobacilli* population (Kim et al. 2010). Studies on the effects of lactose addition on the microbiota communities focus on *Lactobacillus* or

Bifidobacterium changes with the understanding that lactose is the preferred substrate for these genera in colon to produce lactic acid (Molino et al. 2011, Pierce et al. 2006, Pierce et al. 2007). However, our study indicated that the piglets may have produced enough lactase to metabolize lactose in the small intestine and efficiently absorbed lactate on their transit to colon hence minor observable response to diet treatments even when fed high level lactose. The microbiota of colon is affected not only directly by feeding but also indirectly by the metabolites produced and by cross-feeding of microorganisms (Molbak et al. 2007).

The sequencing of colonic digesta in our study resulted in a community structured in which Firmicutes was the most abundant phylum, followed by the phylum Bacteroides. These two phyla made up 88 to 90 % of the bacterial community. The observation is comparable to a study of bacterial community in faecal samples obtained from 10 weeks old healthy pigs raised in 2 separate commercial farms (Kim et al. 2011,Lamendella et al. 2011) in colon, followed by the genus *Prevotella*. As a comparison, *Prevotella* was the most abundance in faecal samples of 10 weeks old piglets. Futhermore, the authors observed an increase in *Clostridia* and decrease in *Prevotella* with age (Kim et al. 2011). It is acknowledged that microbiome in colon and in faeces are not completely the same (Leser and Molbak. 2009,Leser et al. 2002). However, our study indicated that 5 weeks old piglets fed with WP exhibited colon community structure similar to 10 weeks healthy piglets raised in commercial farms.

The abundance of the phyla Bacteroides was affected by experimental diet in which at genus level, WP addition at 60 g/kg decreased the abundance of *Prevotella*. Similarly, gradual decrease in *Prevotella* abundance was reported in the ileum and colon of piglets fed with pectin rich chicory-forage (Liu et al. 2012). Various species of *Prevotella* in human colonic microbiota degrade dietary xylan from cereal grains. The number of coliform (as represented as Enterobacteriacea count) decreased in WP60 and WP120 groups. However, results from Illumina sequencing revealed no affect on the abundance of Enterobacteriaceae family. The improved feed conversion ratio may have supported the generally stable colonic microbiota. Additionally, Shannon-weaver indices from colonic microbiota community indicated unchanged diversity across different feed treatments. Previously, DGGE analyses on colonic samples of post weaned piglets fed lactose resulted in numerical decreased diversity of *Lactobacillus* spp. at 12 % compared to 8% (Molino et al. 2011). The authors suggesting that feeding post weaned piglets higher than 8% might inhibit the growth of this particular group.

In our study, we found Tenericutes as the third abundant phylum in colon when piglets were fed WP at 120 g/kg or higher. Up to the time when this article was written, there are not many available information about this group. It is found in less abundance in faecal samples of 28 d piglets fed with *L. salivarus* (Riboulet-Bisson et al. 2012) and non-human primates (Yildirim et al. 2010). However, it is still unclear whether there is biological significance of alterations of Tenericutes abundance in colon.

In human, undigested lactose frequently related to intolerance symptoms, including gas, gut pain, diarrhoea or constipation. These symptoms often related to production of metabolic toxins as results of anaerobic digestion of lactose not absorbed in the small intestine (Campbell et al. 2010). In this study, which was performed in post weaned piglets, showed that WP feeding up to 240 g/kg did not generate intolerance or enterocolitis symptoms as indicated by the absence of diarrhoea, low concentration of lactic acid in the colonic samples and insignificant changes in the total thickness of mucosa (villus height and crypt depth combined) in the jejenum and colon and lastly the stable colonic microbiota.
The WP addition to piglets weaned at 28 days improved the feed conversion ratio throughout the experimental period (35 days) and did not disrupt the balance and diversity of colonic microbiota.

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Table 1. Composition of experimental diets

Item	WP additions group (g/kg, as fed) treatment groups				
	0	60	120	180	240
Variolac 830	0.00	60.00	120.00	180.00	240.00
Barley	200.00	200.00	200.00	200.00	200.00
Wheat	481.80	411.80	341.70	271.60	201.60
Dehuled toasted soybean meal	167.30	176.80	186.00	195.30	204.50
Animal fat	30.00	30.00	30.00	30.00	30.00
Soy protein concentrate	30.00	32.50	35.00	37.50	40.00
Potato protein	50.00	50.00	50.00	50.00	50.00
L-Lysine HCL	4.05	3.97	3.89	3.81	3.74
DL-Methionine	1.15	1.21	1.34	1.49	1.63
L-Threonine	0.95	0.97	0.99	1.02	1.04
L-Tryptophan	0.34	0.33	0.32	0.32	0.31
Monocalcium phosphate	13.21	12.41	11.61	10.82	10.03
Calcium carbonate, 38% Ca	11.98	11.85	11.71	11.58	11.45
Sodium chloride	5.05	4.18	3.32	2.45	1.58
Phytase (phyzyme XP*400 TPT)	0.13	0.13	0.13	0.13	0.13
Vitamin and mineral premix	4.00	4.00	4.00	4.00	4.00

Table 2. Chemical composition of experimental diets

Item	WP additions group (g/kg, as fed) treatment groups				
	0	60	120	180	240
Dry matter	88.5	89.9	89.9	90.1	90.9
Protein (N*6.25) %DM	21.8	21.5	22.1	21.1	21.8
Fat; %DM	4.8	5.3	4.5	4.5	4.1
Ash, %DM	4.9	5.3	5.6	5.7	6.1
FU (per 100 kg)	113.3	114.1	114.5	113.6	115.9
Calcium (g/kg)	7.38	7.74	8.10	7.87	8.04
Fosfor (g/kg)	5.77	5.74	6.02	5.88	6.06
Valine (g/kg)	10.58	10.54	10.93	10.38	10.56
Cystein+Cystine (g/kg)	3.65	3.52	3.61	3.44	3.54
Methionine (g/kg)	4.85	4.3	4.87	4.63	4.87
Threonine (g/kg)	9.82	9.52	10.1	9.41	9.65
Lysine (g/kg)	15.6	15.3	16.2	15.4	15.5

Table 3. Growth performance of piglets fed the experimental diets

	WP level (g/kg)						
Item	0	60	120	180	240	SEM	*P*-value
ADG, g							
1 to 14 days	272.0	287.7	296.4	301.2	302.7	6.47	0.579
14 to 35 days	800.9	907.3	849.3	856.3	851.2	16.9	0.226
1 to 35 days	590.8	664.4	641.2	629.0	636.7	11.9	0.226
ADFI, g							
1 to 14 days	285.2	290.8	298.6	301.3	307.2	3.89	0.871
14 to 35 days	1232[a]	1202[a]	1083[b]	1129[ab]	1158[ab]	26.3	0.021
1 to 35 days	967.4	954.0	888.8	918.7	940.9	13.9	0.194
Feed conversion ratio, g/g							
1 to 14 days	1.072	1.031	1.021	1.004	1.021	0.001	0.590
14 to 35 days	1.554[a]	1.341[b]	1.294[b]	1.316[b]	1.361[b]	0.046	0.037
1 to 35 days	1.393[a]	1.242[b]	1.203[b]	1.213[b]	1.250[b]	0.034	0.010

Data are presented as least square means (n = 10).

[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

SEM, SE of the mean.

Table 4. Physicochemical characteristics of gastric, caecal and colonic digesta in weaned piglets fed diets with different WP amounts

	WP level (g/kg)						*P*-value	
Item	0	60	120	180	240	SEM	Diet	Diet x segment
DM content, %							0.92	0.58
Stomach	24.29	22.88	24.37	24.17	22.57	0.38		
Distal ileum	7.68	8.67	8.63	9.91	9.22	0.37		
Caecum	10.12	10.12	9.59	9.76	9.53	0.13		
Colon	17.41	16.68	16.29	17.37	17.51	0.24		
pH							0.58	0.23
Stomach	3.61	3.36	3.54	3.78	3.60	0.07		
Distal ileum	6.96	6.93	6.74	6.72	7.03	0.06		
Caecum	5.85	5.80	5.92	5.75	5.92	0.03		
Colon	6.12	6.07	6.29	6.18	6.43	0.06		

Data are presented as least square means (n = 10). SEM, SE of the mean.

Table 5. The amount of lactic acid and volatile fatty acids (mmol/kg) in the digesta from the gastrointestinal tract of piglets fed experimental diets

	WP level (g/kg)						*P*-value	
Item	0	60	120	180	240	SEM	Diet	Diet x segment
Lactic acid							0.107	0.254
Stomach	38.3	52.2	33.2	30.6	33.8	3.86		
Distal ileum	24.3	25.2	33.8	16.2	13.2	3.64		
Caecum	0.32	4.15	1.36	1.47	1.18	0.65		
Colon	0	0	0	0	0	0		
Acetic acid							0.434	0.809
Stomach	0.37	0	0	0	0	0.07		
Distal ileum	0	0.3	0.1	0.35	0	0.07		
Caecum	13.5	14.6	13.6	13.1	13.5	0.25		
Colon	15.9	18.1	15.3	16.1	14.6	0.59		
Butyric acid							0.237	0.562
Stomach	5.54	4.98	4.28	4.53	4.30	0.24		
Distal ileum	6.11	6.87	6.16	10.0	8.97	0.79		
Caecum	75.5	75.3	73.6	80.8	77.5	1.22		
Colon	70.5	69.2	70.3	73.4	68.3	0.86		
Acetic + propionic + butyric acid							0.513	0.670
Stomach	6.04	5.09	4.28	4.63	4.75	0.30		
Distal ileum	6.21	7.17	6.21	10.4	9.06	0.83		
Caecum	119.5	121.1	118.5	127.0	119.4	1.54		
Colon	116.4	116.7	112.3	118.7	106.7	2.14		

Data are presented as least square means (n = 10). SEM, SE of the mean.

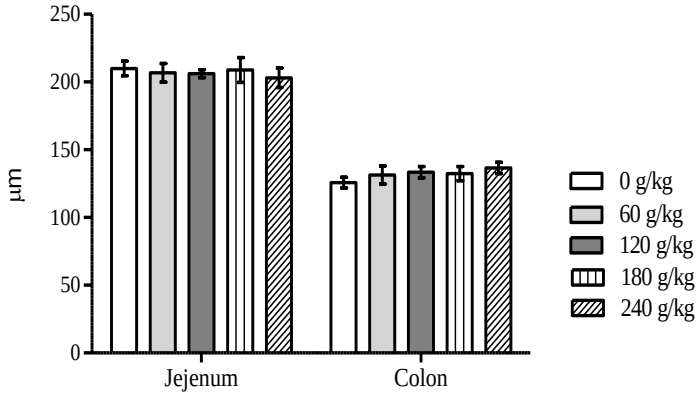
Table 6. Counts of selected microbial populations (log cfu/g of digesta) in digesta from the gastrointestinal tract of piglets fed experimental diets

	WP level (g/kg)						*P*-value	
Segment	0	60	120	180	240	SEM	Diet	Diet x segment
Lactic acid bacteria							0.011	0.703
Stomach	8.58	8.72	8.43	8.55	8.36	0.06		
Distal ileum	8.55^{ab}	8.42^{ab}	8.69^{a}	8.40^{ab}	8.13^{b}	0.09		
Caecum	9.11	9.15	9.13	8.93	8.75	0.08		
Colon	9.50	9.47	9.55	9.26	9.14	0.08		
Yeasts							0.995	0.995
Stomach	2.90	3.18	4.03	3.51	3.72	0.20		
Distal ileum	3.51	3.31	3.78	3.44	3.57	0.08		
Caecum	3.69	3.47	3.98	3.31	3.62	0.11		
Colon	4.00	3.59	4.05	3.32	3.80	0.14		
Enterobacteriaceae							0.554	0.002
Stomach	4.52^{ab}	4.75^{ab}	5.08^{a}	4.33^{ab}	4.43^{ab}	0.13		
Distal ileum	5.36^{ab}	4.71^{ab}	5.69^{b}	5.42^{ab}	5.19^{a}	0.16		
Caecum	6.76^{a}	6.09^{b}	5.99^{b}	5.96^{b}	6.34^{ab}	0.15		
Colon	6.87^{a}	6.06^{b}	6.43^{b}	6.59^{ab}	6.71^{ab}	0.14		
Total anaerobic bacteria							0.068	0.757
Stomach	8.22	8.41	8.29	8.05	8.16	0.06		
Distal ileum	8.40	8.43	8.63	8.11	8.03	0.11		
Caecum	9.26	9.38	9.19	9.01	9.22	0.06		
Colon	9.65	9.72	9.63	9.45	9.46	0.05		

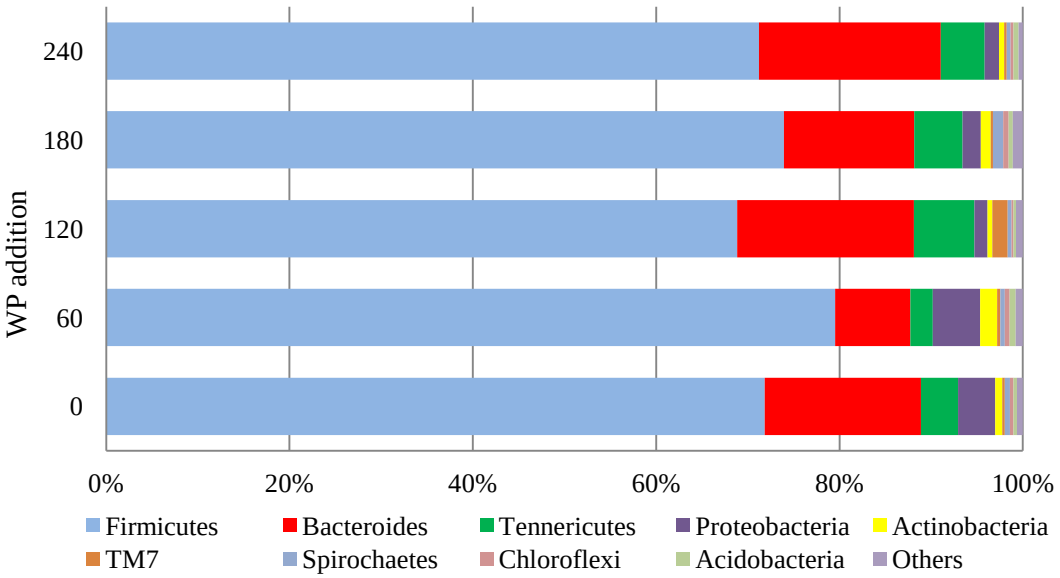
Data are presented as least-square means (n=10).

^{a,b}Within a row, means without a common superscript differ ($P < 0.05$).

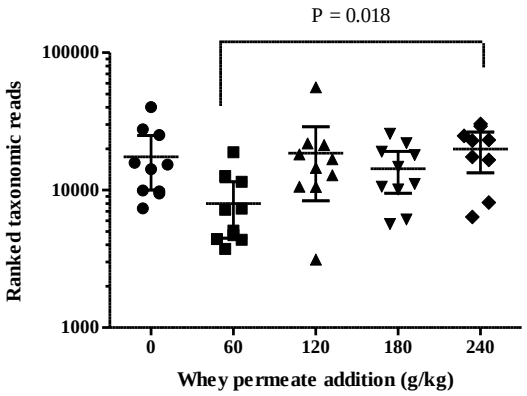
SEM, SE of the mean.


250
200
150
100
50
0
µm
Jejenum
Colon
0 g/kg
60 g/kg
120 g/kg
180 g/kg
240 g/kg

A.


WP addition
240
180
120
60
0
0%
20%
40%
60%
80%
100%
Firmicutes
Bacteroides
Tennericutes
Proteobacteria
Actinobacteria
TM7
Spirochaetes
Chloroflexi
Acidobacteria
Others

B.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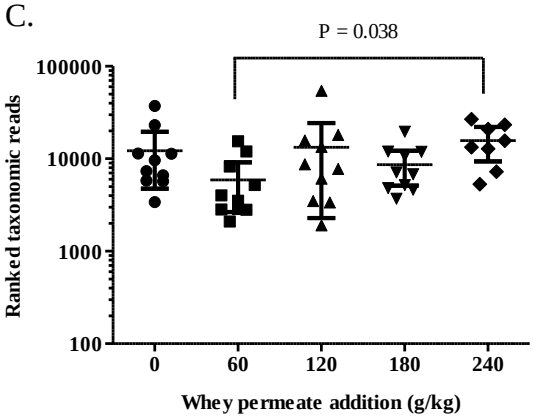
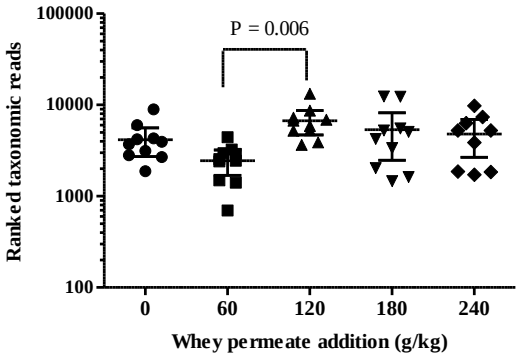
Ranked taxonomic reads
100000
10000
1000
0
60
120
180
240
Whey permeate addition (g/kg)

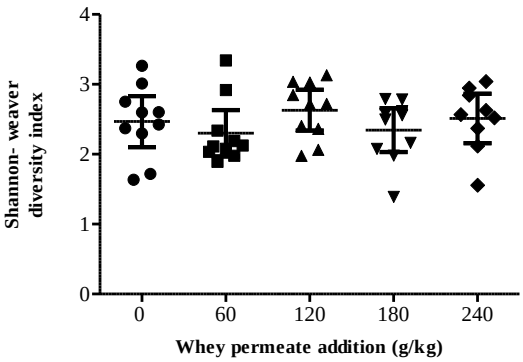
C.


P = 0.038
Ranked taxonomic reads
100000
10000
1000
100
0
60
120
180
240
Whey permeate addition (g/kg)

D.


P = 0.006
Ranked taxonomic reads
100000
10000
1000
100
0
60
120
180
240
Whey permeate addition (g/kg)

E.


Shannon- weaver diversity index
4
3
2
1
0
0
60
120
180
240
Whey permeate addition (g/kg)

Captions for Figures

Figure 1. Total thickness of mucosa (villus height + crypt depth) in the jejenum and colon of piglets fed with different levels of WP

Figure 2. Microbial communities in colonic digesta of piglets fed with different levels of whey permeate (A). Relative abundance at phyla level expressed as ranked taxonomic reads: A1. 0 g/kg; A2. 60g/kg; A3. 120 g/kg; A4.180 g/kg; A5. 240 g/kg; (B). Within the phylum Bacteroides and (C). Genus *Prevotella*; (D).Tenericutes; (E). Shannon-weaver diversity index. Bar on each treatment group was presented as least square mean ± 95% Cl. Means with $P < 0.05$ (Kruskal-Wallis) were different.

Supplementary Material

Table S1. Sequences of hexameres attached as barcodes to primers in building the amplicon library for next generation sequencing on Illumina Hiseq

Barcode no.	5' - 3'	Barcode no.	5' - 3'
1	ACACAC	26	ACGCAG
2	ACAGTC	27	ACTGCG
3	AGCTAC	28	AGACAG
4	AGCAGC	29	AGAGCG
5	AGAGAC	30	AGCACG
6	AGACGC	31	AGCGAG
7	ACTGAC	32	AGTATG
8	ACTCGC	33	ATAGTG
9	ACTATC	34	ATCGCG
10	ACGTAC	35	ATCTAG
11	ACATGC	36	ATGATG
12	ACGAGC	37	ATGTCG
13	ACGCTC	38	TACAGC
14	AGCGTC	39	TACGAC
15	AGTCAC	40	TAGATC
16	ATACTC	41	TAGCAC
17	ATATAC	42	TAGTGC
18	ATCATC	43	TATCGC
19	ATCGAC	44	TATGTC
20	ATCTGC	45	TCACGC
21	ATGCAC	46	TCAGAC
22	ACACTG	47	TCTAGC
23	ACAGAG	48	TCTCAC
24	ACATCG	49	TGACAC
25	ACGACG	50	TGAGTC

Table S2. The most abundance phylotypes as indentified from Illumina Hiseq sequencing

Rank	Annotation based on Greengenes database (Kingdom; Phylum; Class; Order; Family; Genus)	Relative abundance*
1	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; *Clostridium*	13.86
2	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae	7.39
3	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; *Prevotella*	5.29
4	Unclassified**	2.34
5	Bacteria; Firmicutes; Clostridia; Clostridiales	2.29
6	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Oscillospira*	1.63
7	Bacteria; Tenericutes; Mollicutes; RF39	1.50
8	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales	1.42
9	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	1.29
10	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus*	1.14
11	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Ruminococcus*	1.11
12	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Roseburia*	0.89
13	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Blautia*	0.66
14	Bacteria; Firmicutes; Clostridia; Clostridiales; Catabacteriaceae	0.63
15	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Faecalibacterium*	0.42
16	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*	0.44
17	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae	0.35
18	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Coprococcus*	0.34
19	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Ruminococcus*	0.31
20	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Clostridium*	0.32
21	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Lachnospira*	0.23
22	Bacteria; Tenericutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; *Bulleidia*	0.20
23	Bacteria; Tenericutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae;	0.22
24	Bacteria; TM7; TM7-3; CW040; F16	0.17
25	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; *Treponema*	0.17
26	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides*	0.16
27	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	0.16
28	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; *Parabacteroides*	0.14
29	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	0.11
30	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Butyrivibrio*	0.13

* Number of total ranked taxonomic reads for respective phylotype across all animals and was normalized to 100000

** Unclassified = consensus reads which did not result in any hits based on Greengeen database.

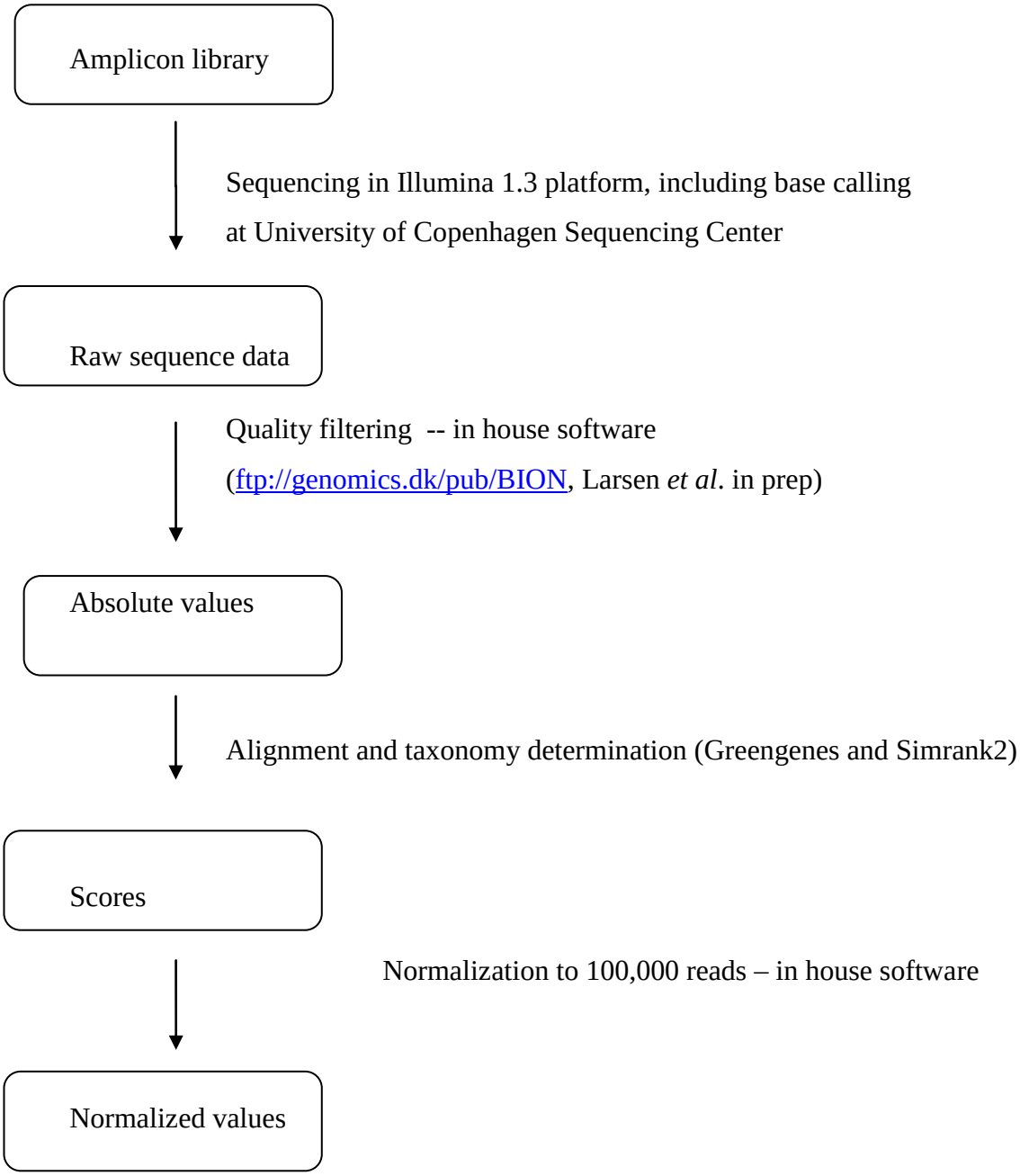


Figure S1. Processing of sequence outputs generated from the Illumina Hiseq sequencing

Paper 2. Submitted for publication in International Dairy Journal

Unsupplemented whey permeate for the selection of lactic acid bacteria with probiotic characteristics

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Running title: unsupplemented whey to grow lactic acid bacteria with probiotic potential

Keywords: probiotic, whey permeate, lactobacilli

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Abstract

The diversification of whey permeate applications to improve sustainability of cheese production is ongoing. The aim of the study was to screen and cultivate lactic acid bacteria (LAB) with probiotic characteristics in unsupplemented whey permeate. Thirtyone out of 121 lactic acid bacteria isolates of different origins were capable to grow in unsupplemented whey permeate. The final selections: three *L. plantarum* and one *L. rhamnosus* inhibited *E. coli* F4, *Streptococcus suis*, *Listeria monocytogenes, Salmonella* Typhimurium and *Clostridium perfringens*, survived to a maximum of 2% porcine bile and attached to IPEC-J2 cell line. These 4 isolates were susceptible to antibiotics, had potential of producing 35.6 mmol kg^{-1} lactic acid and reaching cell density up to 10^9 CFU mL^{-1} in 24 h. The study showed applicability of unsupplemented whey permeate as growth medium to obtain a product consisted of combined viable potential probiotics and lactic acid for food and feed applications.

1. Introduction

Whey permeate is a bulky by-product in dairy processing. It contains an abundant amount of lactose (up to 52 g L^{-1}) as well as some residual nitrogenous materials and salts. Diversified utilization of whey permeate is attractive to the dairy processing sector to obtain value added products with consequent improvement of production sustainability (Panesar, Kennedy, Gandhi, & Bunko, 2007).

The majority of value added application of whey permeate thus far are for lactic acid and biomass production and for feed additive (Aeschlimann & Vonstockar, 1989; Amrane, 2005). As a feed additive, whey permeate reportedly improves the growth performance (average daily weight gain and average daily feed intake) of post-weaned piglets (Molino et al., 2011; Naranjo, Bidner, & Southern, 2010; Pierce et al., 2006). Furthermore, whey permeate consumption has been claimed to potentially improve intestinal health by reduction of gut pH, proliferation of *Lactobacilli* number in the proximal colon, increased the amount of short chain fatty acids and decrease coliform population in fecal samples (Molino et al., 2011; Pierce et al., 2006).

Probiotic is defined as "live microorganisms which when administered in adequate amounts confer health benefits on the host" (FAO/WHO, 2001). Several characteristics are essential in the selection of potential probiotics. These include the ability to: survive harsh conditions in the GI tract, limit the growth of potentially pathogenic microorganisms and adhere to the surface of intestinal cells (Casey et al., 2004; Guo, Kim, Nam, Park, & Kim, 2010; Jacobsen et al., 1999; Lähteinen et al., 2010). Probiotic applications in food and feed also require safety assessments including bacterial resistance to antibiotics. In European countries, a guideline to determine whether a particular bacterium is safe for food or feed application is available (EFSA 2008).

There have been few reports in the utilizations of whey permeate with supplementations as a growth medium to cultivate previously identified lactic acid bacteria (Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010; Mondragon-Parada, Najera-Martinez, Juarez-Ramrez, Galindez-Mayer, Ruiz-Ordaz, Cristiani-Urbina, 2006). However, to the best of our knowledge, there is no study about using this low cost dairy ingredient without any supplementation for culturing newly obtained LAB isolates with probiotic characteristics. A final product which consisted of good numbers of viable probiotics, organic acid metabolites, and residual lactose would be a great value improvement to the whey permeate. For this

particular study, the objectives were to i) screen LAB isolates which grew in unsupplemented whey permeate and showed probiotic characteristics *in vitro*, including their ability to: inhibit selected human and/or animal pathogens; to survive porcine bile and gastric juice, and adherence to intestinal porcine cell line; and ii) apply final probiotic selections in flask fermentation of unsupplemented whey permeate.

Materials and methods

2.1. Growth media preparation

Whey permeate powder (Variolac 830®, Arlafoods, Denmark) was used as growth media for the screening process. Media was prepared by reconstituting whey permeate powder with deionized water to obtain a concentration of 70 g L^{-1}. The solution was pasteurized at 65 °C for 30 min. Direct plating after pasteurization of reconstituted whey permeate showed a complete removal of intrinsic microflora (data not shown). Such media hereafter will be written as Reconstituted Whey Permeate (RWP).

1.2. *Lactic acid bacteria isolations and growth in RWP*

A total of 121 LAB isolates were included in this study. The sources of the isolates were fermented liquid feed in the farm and in the lab (62 isolates), piglet feces (18 isolates), piglet colon and ileum (3 isolates), fermented and nonfermented dairy products (15 isolates), fermented vegetables (9 isolates), feed supplement (8 isolates), fermented meat (3 isolates), milking machine (2 isolates) and 1 isolate from human intestine. A probiotic isolate, *L.acidophilus* DSM 13241 and two bacteriocin producers *L. plantarum* DDEN 11006 and *Pediococcus acidilactici* NRRL B-5627 were also included in the study (Bernbom , Licht, Saadbye, Vogensen, & Nørrung, 2006).

The ability of each isolate to grow in whey permeate was evaluated. Overnight LAB were prepared from respective glycerol stock by inoculating tubes containing 5 mL MRS broth with 50 µL thawed cell suspension. The tubes were incubated at 37 °C for 16 h. The overnight isolate was added to inoculate RWP at the 1% (v/v) rate. RWP was supplemented with filter (d=0.22 µm) sterilized bromophenolblue (0.01 g L^{-1})(Missotten et al., 2009). An amount of 200 µL inoculated RWP was transferred into sterile 96-well plates. The plates were covered and incubated at 37 °C under aerobic condition. Cell densities (OD_{620}) and colour changes were then observed at 0, 8, 12, 16 and 20 h incubation time. As pH drops, bromophenolblue-RWP changed colour gradually from blue to light blue, finally becoming colourless at pH 4.8 or below. Colour changes were evaluated by assigning arbitrary numbers

from 0 (no change) to 5 (colourless). Isolates receiving a score of 3 and/or above at 20 h incubation time, were selected for further screening.

2.3 Identification of isolates to species level

Thirty one isolates were selected from the screening process in RWP and were further identified by sequencing the amplified region of 16S rRNA gene. These LAB, which included both newly isolated and previously identified strains, were grown in MRS (Oxoid) broth for 20 h at 37 °C under aerobic condition and were spread on MRS plates. Genomic DNA was extracted by boiling single isolates in MilliQ water to lyse the bacterial cells. A fragment (*ca*. 950 bp) of the 16S rRNA gene was amplified by PCR using the following universal primers: 519 forward (5'- CCA GCA GCC GCG GTA ATA C - 3') and 1509 reverse (5'- GTT ACC TTG TTA CGA CTT CAC - 3') primers (Edwards, Rogall, Blocker, Emde, & Bottger, 1989)(Eurofins MWG Operon, Ebersberg, Germany). The PCR run conditions were 94 °C for 3 min 15 s, 34 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min and 30 s, followed by 72 °C for 10 min. PCR products were purified by using High Pure PCR Product purification kit (Roche Diagnostic, Mannheim, Germany). Purified PCR products were included in cycle sequence reactions prepared with BigDye Terminator mix (Applied Biosystems) and primers 1509, 1392 reverse (5' TGA CGG GCG GTG TGT ACA A 3'), 1054 forward (5' CAT GGY YGT CGT CAG CTC GT 3'), and 1054 reverse (5' ACG AGC TGA CGA CRR CCA TG 3') (Eurofins MWG Operon, Ebersberg, Germany) in a T3 Thermocycler (Biometra). PCR for sequencing run conditions were 96 °C for 3 s, 50 °C for 15 s and 25 cycles of 60 °C for 4 min. Sequencing was performed with an ABI3700 Capillary DNA Sequencer (Applied Biosystem Inc., Foster City, CA, USA). The chromatograms were manually assembled in BioNumerics version 4.5 (Applied Maths, Belgium).

Isolate identification to the species level was determined based on >97% identity to 16S rRNA gene reference sequence obtained from the GenBank public database (http:www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned for the construction of phylogeny neighbour joining with ClustalX (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). To avoid unnecessary clustering, only four representatives (out of 14) of *L. plantarum* were included in the multiple alignment steps (isolates 23, 54, 104, and 109) and in the drawing of the phylogenetic tree. Bootstrap analyses were performed on 1000 re-sampling of the sequence. The tree files were drawn using the Molecular Evolutionary Genetics Analysis (MEGA 4) program (Tamura, Dudley, Nei, & Kumar, 2007).

2.4 Nucleotide sequence accession numbers

The nucleotide sequences found in the present study (28 sequences) have been assigned Genbank accession numbers JX409626 – JX409653. Genbank accession numbers of three previously identified isolates were also listed (Table 1).

2. 5. Probiotic selection criteria

Selection of LAB with probiotic potential was firstly based on the ability to inhibit the growth of Gram-positive and Gram-negative pathogen indicators. The second criterion was the ability to survive gastrointestinal conditions and the last was the susceptibility to antibiotics. Susceptible isolates were further evaluated for their ability to attach to intestinal epithelial cell line (Fig. 1).

2.5.1. Detection of antimicrobial activity

Five indicator bacteria considered potentially pathogens to human or pigs were included in the study: *Listeria monocytogenes* CCUG15526, *Salmonella enterica* ser. Typhimurium 9616368-3 (*S.* Typhimurium), *Streptococcus suis* NCTC 10234 and *Clostridium perfringens* NCTC10240. The fifth pathogen, *Escherichia coli* 9910045-1:0149ST2,LT,F4ac (hereafter will be written as *E. coli* F4), was first isolated at the Danish Veterinary Insitute (Frydendahl, Imberechts, & Lehmann, 2001). Working cultures of respective indicator strain were prepared by transferring a single isolate from Blood Sheep agar (Oxoid) into Brain Heart Infusion (BHI, Oxoid) broth. The tubes were incubated at 37 °C for 24 h under aerobic conditions except for *C.perfringens* which was incubated anaerobically. Aliquots of the refreshed culture were kept in glycerol (25 % v/v) at -80 °C until use.

Analyses of antimicrobial activities started with growing indicator pathogen on Blood Sheep (Oxoid) agar and refreshed in Brain Heart Infusion (BHI, Difco) broth media. The assay plates were prepared by inoculation of 50 mL 45 °C BHI agar with respective pathogen to reach approximately 10^6 CFU mL^{-1}. The inoculated agar was poured into a plate (d= 145 mm) (Greiner Bio-One, Frickenhausen, Germany). Wells (d= 6 mm) were then created on the solidified agar.

The 31 RWP-growing LAB isolates were refreshed from -80 ^{0}C stock in MRS broth incubated for 12 h at 37 °C. The second transfer was used to inoculate 15 mL MRS to reach OD_{600} values of 0.05 prior to incubation at 37 °C for 12 h. Fermentation broth was centrifuged at 180 x g (Hettich, Germany) for 15 min to separate cell-free supernatant from

cell pellet. Cell pellet was resuspended in PBS buffer (pH 7.0). The antimicrobial activity was detected by diffusion assay method. A 70 µL of fermentation broth (fraction A), cell-free supernatant (fraction B), and resuspended cell pellet (Fraction C) were respectively added into wells on the plates with the pathogens. All plates were incubated at 37 °C for 24 h under aerobic conditions, except for *Clostridium perfringens* plates which were incubated under anaerobic conditions. Clear inhibition zones surrounding the wells were measured to determine antimicrobial activity of LAB isolates. Each plate included a negative control well which was filled with sterile MRS broth and a positive control well which was filled with fermentation broth of *L. plantarum* isolate 109. Nine LAB isolates showing broad antimicrobial activity were refreshed from the -80 °C stock by inoculating MRS broth followed by incubation for 12 h at 37 °C. The second transfer was performed in RWP media, which was later used for inoculating 15 mL RWP to reach an OD_{600} of approximately 0.05 prior to incubation at 37 °C for 12 h. The evaluations of the in vitro antimicrobial activities of these 9 LAB isolates were performed in triplicate.

2.5.2. Tolerance to gastrointestinal conditions

The tolerances of LAB isolates to synthetic gastric juice acidic pH and porcine bile salts were evaluated according to (Casey et al., 2004) with modifications. Briefly, survival in synthetic gastric juice was evaluated by resuspending overnight grown and washed LAB isolates in synthetic gastric juice to reach an initial population of *ca.* 10^8 CFU mL^{-1}. The mixture was incubated at 37 °C under aerobic conditions. Samples were obtained at 30 and 180 min, serially diluted and enumerated on MRS agar. Surviving colonies were counted and compared against the control group grown in MRS broth. The resistance to gastric juice was expressed as percentage (%) survival of the isolate at given sampling time. The assays were performed in duplicate.

The tolerance to porcine bile was evaluated by streaking overnight grown isolate containing *ca*. 10^6 CFU mL^{-1} on MRS plates with different porcine bile concentrations (0,3; 0,5; 1; and 2% w/v). Streaked plates were incubated at 37 °C for 72 h in anaerobic jar. The assays were performed in duplicate. The tolerance to porcine bile was expressed as the highest porcine bile concentration in the MRS plate at which growth was observed.

2.5.3. Susceptibility to antibiotics

Eight selected and identified isolates were tested for antibiotic susceptibility according to EFSA and Clinical and Laboratory Standards Institute (CLSI) guidelines (EFSA, 2008;

EFSA, 2010). *Enterobacter faecium* 94 was not included in further evaluations based on documentation by EFSA that the genus *Enterococcus* spp. is not among the proposed organisms to receive Qualified Presumption of Safety (QPS) status (EFSA, 2007). Briefly, respective selected LAB isolate was grown for 20 h at 37 °C in Mueller Hinton broth supplemented with lysed horse blood (Oxoid). An inoculum equivalent to 0.5 McFarland standard was added into each well on a 96-well plate containing a range of antibiotic. The Minimal Inhibitory Concentration (MIC) value was defined as the lowest concentration in the test-range with no visible growth of the tested LAB isolate. Specific MIC values (breakpoints) for determining the susceptibility of *Lactobacillus* isolates to antibiotics were provided in an EFSA guideline. At the time of our experiment, *Weissella* was not part of EFSA's working group's evaluation list. Therefore, breakpoints for *Leuconostoc* were applied to determine the susceptibility of *Weissella* isolates to antibiotics.

2.5.4. Adhesion of LAB isolates to IPEC-J2 cell line

The IPEC-J2 cell lines (Intestinal Porcine Epithelial Cell Jejunum) were originally collected as a non-transformed intestinal cell line from jejuna epithelia isolated from a neonatal, non suckled piglet. The cells were maintained as previously described (Schierack et al., 2006). Briefly, a cell line from a -80 °C collection was regrown in 15 mL Dulbecco's modified eagle medium (DMEM:F12 = 1:1, Merck, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Merck, Germany), 2 mmol L^{-1} L-Glutamine (Sigma), 1 mmol L^{-1} pyruvate, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Sigma) and maintained at 37 °C under 5% CO_2 atmospheric level. Cell growth media were replaced every second day and monolayer cell lines were routinely subcultured every 7 days. Prior to studies, cell lines were routinely tested to confirm the absence of mycoplasma.

Four selected LAB were evaluated for their ability to adhere to IPEC-J2 monolayer cell lines. The cells were seeded at a concentration of 1.06×10^5 cells per well in 4-well culture slides (BD Falcon, Sparks, MD, USA) and grown into confluence (2-3 days) in growth media. The day before the addition of selected LAB cells, growth media were replaced with media without antibiotic supplementation. LAB isolates grown overnight were pelleted prior to washing twice with PBS. LAB cells were then resuspended in the antibiotic-free IPEC-J2 growth media. LAB cells were added into wells containing confluence IPEC-J2 cells at a final concentration of 1.05×10^7 per well. The inoculated culture slides were incubated for 2 h at 37 °C under 5% CO_2 atmospheric level. Monolayers were washed twice with PBS buffer

prior to fixation for 30 min at room temperature with freshly made 4% (w/v) paraformaldehyde in PBS. Fixed monolayers were washed twice with PBS, air-dried and stained with propidium iodide provided as part of the *LIVE/DEAD® BacLight™* Bacterial Viability Kits (Invitrogen, Carlsbad, CA, USA). Adherence of each LAB isolate to IPEC-J2 cell lines in 4 wells was observed under the 100 x objective magnification lens of a Zeiss optical binocular microscope coupled with an Axio camera and used with a computer supported by the AxioVision rel 4.8 software. Ten random areas per well were observed. Arbitrary units from 0 to 5 were assigned to LAB cell counts as follows: 1 (up to 50 LAB cells), 2 (50 to 100 cells) 3 (100 to 150 cells), 4 (150 to 200 cells), and 5 (more than 200 LAB cells). Isolate was considered well attached if 200 colonies/area of observation were observed. The area of observation was 0.045 mm^2.

2.6 Growth profile of selected isolates

Four final selections were further studied to obtain their growth profiles in 100 ml MRS or RWP media. Isolates were prepared by refreshing -80 °C culture collections twice and then added to either MRS or RWP media as inoculant. An initial OD_{600} of 0.05 and obtained and this was followed by incubation at 25 °C or 37 °C for up to 32 h with gentle agitation (50 rev min^{-1}). Samples were taken at time intervals for optical density (OD_{600}) and pH measurements. Samples at 24 h incubation were analyzed for microbiology counts on MRS plates. Broth samples from 0, 8, 16, and 24 hours were filtered through 0.2 µm (Millipore, Ireland) and stored at -20 °C for lactic acid and short chain fatty acids analysis. Lactic acid, acetic acid, and succinic acid amounts were analyzed in GC system as previously described (Canibe & Jensen, 2007). Residual lactose was calculated based on the stoichiometry of homolactic fermentation.

2.7 Statistical analyses

The results of antimicrobial activities were expressed as the means and standard deviation (S.D). The scores of LAB attachment to IPEC-J2 cells were expressed as the median and interquartile range. The percentage survival of LAB in gastric juice were analysed with one-way ANOVA with Tukey post test using GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA). P-values less than 0.05 were regarded as statistically different.

3. Results and discussion

3.1. Initial screening by growing isolates in Reconstituted Whey Permeate (RWP) media

In the present study, unsupplemented Reconstituted Whey Permeate (RWP) was utilized to screen lactic acid bacteria isolates obtained from various natural environments. The only carbohydrate source available in the growth media was lactose. This study found that 70 g L^{-1} unsupplemented RWP was sufficient for few (31 out of 121) of the isolates to proliferate (Table 1). These 31 isolates exhibited colour changes from deep purple to light blue and yellow which corresponded to pH value of 5.0 or lower in less than 20 hours (Fig. S1). Three isolates obtained from feed additive product (isolate 93, 94 and 95) and isolates from whey permeate (isolates 99, 100, and 101) exhibited pH lower than 4.0 and OD values greater than 1.0. Isolates obtained from fermented liquid feed (18 isolates) made up the most of the selected group. It was previously reported that MRS-bromophenolblue helped in selecting LAB with desirable acidification rate. Furthermore, the authors found that 10 strains which reduced the pH to 4.9 or lower within 24 h exhibited probiotic characteristics *in vitro* (Missotten et al., 2009).

Growth of different probiotics in soy milk improve after the addition of lactose, suggesting that all tested probiotic were capable of uptaking and digesting lactose in soy based medium (Ding & Shah, 2010). On the contrary, a probiotic strain *L. acidophilus* DSM 13241 which was included in this study did not show growth in RWP (data not shown) and was not selected for further evaluations. Reports on growing LAB in whey based media always include the need for supplementation, mostly in the form of nitrogen source (Amrane, 2005; Mondragon-Parada et al., 2006). This indicate that probiotic like *L. acidophilus* DSM 13241 requires enrichment to be able to grow in whey permeate medium.

3.2 Identification of screened isolates

Identification based on 16S rRNA gene sequencing confirmed that the 31 screened isolates belong to 4 different genera: *Lactobacillus*, *Weissella*, *Pediococcus*, and *Enterococcus* (Table 1). Missotten et al. (2009) identified LAB isolates from fermented liquid feed with desired acidification rate as belonging to *Lactobacillus*, *Streptotoccus*, *Pediococcous* and *Bifidobacterium*. Furthermore, Ayeni et al. (2011) identified *Lactobacillus* and *Weissella* as the results of isolating probiotics from whey. These genera are commonly found in dairy and fermented products indicating that the source and method of isolation influenced which genera were found during the screening process.

We found 13 isolates obtained from fermented liquid feed as *L. plantarum* which was consistent with studies that isolated LAB from fermented liquid feed under similar conditions (Canibe, Hojberg, Badsberg, & Jensen, 2007; Olstorpe, Lyberg, Lindberg, Schnurner, & Passoth, 2008). The authors found *L. plantarum* as one of the most frequently isolated species. Among LAB strains, *L. plantarum* is the most versatile and flexible species. It contains a diverse sugar utilization system derived from clustering related transporter, metabolic enzymes and other regulatory proteins on a lower GC content region (Zhu, Zhang, & Li, 2009).

The phylogenetic tree (Fig. 2) demonstrated that 4 of the isolates showing capacity to grow in RWP and identified as *L. rhamnosus* were related to *L. paracasei* subsp. *tolerans* ATCC 25599^{T}. However, neither relatedness nor similar growing properties suggested shared probiotic characteristics (Missotten, et.al, 2009), urging that each strain needed to be evaluated individually.

3.3. Evaluations of probiotic characteristics

The ability to inhibit the growth of pathogenic bacteria is considered as one of important criteria for screening probiotic (FAO/WHO, 2001). LAB strains originating from various sources show capacities to inhibit pathogens (Guo et al, 2010; Lähteinen et al., 2010; Missotten et al., 2009). The 31 RWP-growing isolates were evaluated for their ability to inhibit human and animal pathogens. Measurements of inhibition zones indicated that the antimicrobial activities varied among LAB isolates.

A total of nine isolates showed a broad range of inhibitions after grown in MRS: included four *L. plantarum* strains (isolate 15, 54, 65, and 109), *L. casei* 21, *L. rhamnosus* 93, *W. paramerenteroides* 17, *W. viridescens* 19, and *Enterococcus faecium* 94 (Table S2). Alokami et al. (2000) reported that lactic acid at pH 4.0 disrupted outer membrane of Gram-negative bacteria, including *E. coli* and *Salmonella* Typhimurium. However, our study showed that among isolates that reached pH values lower than 4.0, only some *L. plantarum, L. rhamnosus* and *Weissella* strains inhibited both *E.coli* F4 and *S.* Typhimurium, other strains inhibited only one of the pathogens, and 3 *L. rhamnosus* strains did not show any inhibitions (Table S2). This observation indicated that even when it is important, sufficient acidity did not always go hand in hand with inhibition of Gram-negative pathogens.

Four of the *L. plantarum* strains behaved differently against Gram-negative and Gram-positive pathogens, indicating that the antimicrobial capacities were strain dependent which

were in agreement with previous studies (Jacobsen et al., 1999; Missotten et al, 2009). The 9 selected LAB strains showed less inhibition capacities after being grown in RWP when compared to inhibitions after grown in MRS (Table 2). This could be due to lower OD values which provided less number of cells to metabolize lactose to produce lactic acid. Nonetheless, *Strep*. *suis* was inhibited by all 9 isolates and *E. coli* F4 was inhibited by 8 selected strains. As expected, *L. plantarum* isolate 109, a pediocin producer, retained its ability to inhibit *Listeria monocytegenes* (Bernbom et al., 2006).

Fractioning fermentation broth into neutralized resuspended cells or supernatant lessened or removed the inhibition capacities of RWP- grown isolates (Table S2). The results suggested that the combination of lactic acid and the bacterial cells in the fermentation broth were responsible for the inhibition capacities. Similarly, the ability to inhibit *Salmonella* and *E. coli* by selected LAB disappeared after applying only supernatant or when the pH of the supernatant was adjusted to 6.0 (Casey et al., 2004; Guo et al., 2010; Lähteinen et al., 2010). Our results indicated that these 9 selected strains possessed the ability to inhibit Gram-positive and Gram-negative pathogens given there were sufficient acidity due to the presence of lactic acid and viable cells.

In order to function as probiotic in the intestinal site, orally delivered bacteria need to survive transport in the upper gastrointestinal tract (GIT) passage (Casey et al., 2004; Jacobsen et al., 1999; Lähteinen et al., 2010). The nine selected isolates were evaluated for their ability to survive simulated gastric juice and different concentrations of porcine bile. Eight out of 9 isolates showed survival at a range of porcine bile up to 2% (Table 3). Bile acids concentration measured in the human intestines typically ranges from 10 mmol L^{-1} in the proximal intestine to 2 mmol L^{-1} in the distal site. A 1% w/v bile acid solution consists of approximately 26 mmol L^{-1} total bile acids (Edwards & Slater, 2009). The ability to sustain 0.3% bile conjugated bile acid or higher suggests survival of bacteria in the intestinal environment (Casey et al., 2004). Similar results, in which LAB strains that were inhibitory against *Salmonella* were found to be resistant to porcine bile up to 5% (Casey et al., 2004). Survival rates in gastric juice varied among the 9 isolates. Overall, the results showed that selected LAB isolates were more sensitive to simulated gastric juice than to porcine bile, which was consistent with a study which reported a *L.pentosaceus* strain which survived 5% porcine bile but were sensitive to simulated gastric juice (Casey et al., 2004). In addition, we observed that isolates with poor or no resistance to porcine bile, were not able to survive gastric juice as shown by *L. rhamnosus* 93, *Enterococcus faecium* 94, and *L. plantarum* 109.

Weissella isolates exhibited greatest survival rates in artificial gastric juice followed by *L. plantarum* isolate 65 and 54.

A comprehensive understanding of how bacteria tolerate bile acid is still unclear. One of the mechanisms being proposed suggests that commensal gastrointestinal bacteria endure bile acid stress partially by deconjugation process catalyzed by bile salt hydrolase (BSH) enzymes (Begley, Hill, & Gahan, 2006). Resistance to bile acid by the 6 selected LAB in this study suggested the potential to adapt to the intestinal environment and stay viable when bile acid is present.

At this stage of the experiment, we decided to remove *E. faecium* isolate 94 from further investigations. Enterococci are members of the LAB community, commonly found in fermented products and part of the GIT microbiota of humans and animals. However, they remain controversial because most species harbor a series of virulence factors and have been associated with a number of human infections (EFSA, 2010, Foulquie Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). The susceptibility of the remaining 8 isolates to antibiotics was then tested. *L. casei* obtained from piglet feces was resistant to tetracycline. Both *Weissella,* which were evaluated based on the breakpoints of *Leuconostoc* were resistant to tetracycline (Table S3). These results left us with 4 *Lactobacillus* isolates for further assessments.

All 4 lactobacilli isolates resulted in median scores of 5.0 (attached well) (Table 3 and Fig. S4). With a P value = 0.1664, the medians were not different between the isolates. Similarly, lack of significant differences in ability to adhere to Caco-2 cell line was observed (Tuomola & Salminen, 1998). However, a separate study reported *Lactobacillus* spp. did exhibit different adherance abilities to IPEC-J2 cell line (Larsen, Nissen, & Willats, 2007). Seemingly contradictory observations may be related to the different ratio of lactobacilli to eukaryote cell numbers during the analysis. Adhesion of *Lactobacillus* is concentration-dependent and with no saturation level up to 20:1 (lactobacilli : eukaryote cell) mixture (Tuomola & Salminen, 1998). In our study, a 100:1 (lactobacilli:eukaryote cell) mixture were used, with the results that LAB cells attached very well to the cell line. Comprehensive all around probiotic properties rarely exhibited by one particular strain (Lähteinen et al. 2010) and the final decisions need to be based on overall selection criteria. In our case, we chose that sensitivity towards antibiotics was more crucial than the ability to survive simulated gastric juice and porcine bile. Therefore, we decided to keep *L. rhamnosus* 93 but excluded

E. faecium 94 and both of the *Weissella* strains. The final 4 selections then included 3 *L. plantarum* strains, and 1 *L. rhamnosus*.

3.4. Growth profile of 4 selected LAB isolates

Selected isolates showed different growth rates when grown in MRS or RWP respectively (Table 4 and Fig. 3). In general, MRS media and incubation at 37 °C provided better growth for all selected LAB isolates. In comparison, growing in RWP showed much lower maximum growth rate for these 5 isolates, especially when the fermentation continued at 25 °C. However at 37 °C OD values reached in the range of 0.5 – 1.4 which corresponded to cell densities between 1.0 x 10^8 and 1.1 x 10^9 CFU mL^{-1}. These results were comparable to or even better than a previous study which grew different lactic acid bacteria and propionic bacteria in supplemented whey permeate. In that study, yeast extract supplementation resulted in OD_{600} values between 0.6 – 1.6 for lactobacilli after 24 h incubation (Hugenschmidt et al., 2010). Furthermore, preparing inocula in the same medium used for the growth studies gave relatively short lag phase (2 h or less) both in MRS and RWP (Fig. 3). *L. rhamnosus* 93 converted lactose to lactic acid at a higher rate compared to other isolates. After 24 h incubation in RWP, it produced 35.6 mmol kg^{-1} lactic acid. All 4 isolates grown in RWP under our experimental conditions were homolactic as no acetic acid was produced (data not shown). Studies which concentrated in improving conversion rate of lactose to lactic acid reported a much higher lactic acid concentration (450 mmol kg^{-1}) after growing *L. helveticus* in whey permeate (Aeschlimann & von Stockar, 1990), even a 100 % conversion rate (Mondragon-Parada et al., 2006). However, supplementation of whey permeate with as high as 2.5% yeast extract and buffered the growth media at 6.0 were necessary. This approach could be relevant for further study when one of the 4 final selections will be applied at production scale.

The growth studies of selected LAB confirmed that at 37 °C, the bacteria grew in RWP, produced lactic acid and lowered the pH while maintaining viability during the 24 h period (Table 4, Fig. 3). The highest maximum specific growth rates (μ_{max}) obtained in our study (0.030) was slightly lower than a previous study which reported a μ_{max} of 0.035 from growing a *L. casei* strain in a batch fermentation for 66 h in a supplemented (0.25 g/L yeast extract) whey permeate medium (Mondragon-Parada et al., 2006). This showed, that the final selections from our study had the potential for a larger specific growth and lactose to lactic

acid conversions rate, given optimization of growth conditions. However, to meet our objectives, we would prefer to have residual lactose in the final product. After 24 h, we observed that there was a substantial amount of residual lactose (46- 51 g L^{-1}) (data not shown. The effect of mixing selected lactobacilli from this study, for example *L. rhamnosus* 93 and one of the *L. plantarum* isolates as multi-strain inocula to obtain fermented RWP with probiotic properties is still to be tested.

4. Conclusions

Our study showed that screening LAB using Reconstituted Whey Permeate (RWP) without any supplementations resulted in 4 lactobacilli which exhibited probiotic characteristics *in vitro*. The final product of flask fermentation using monoculture of the final selections consisted of viable potential probiotics, lactic acid as metabolites, and residual lactose.

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Tables.

Table 1 List of 31 isolates that showed growth in whey media, their natural sources and the results of identification to species level

Isolate	GenBank ID[§§]	Natural source	Reference	OD_{600} in RWP	pH in RWP*	Best hit from blast[§]	Similarity[§] (% identity)
4	D16551.1	Cheese	(Collins & Martinez-Murcia, 1991)	0.45	4.17	*L. casei*	100
10	JX409626	Fermented whey permeate	This study	0.51	4.37	*L. plantarum*	100
12	NR_040812.1	Fermented sausage	(Collins et al., 1993)	0.26	4.40	*Weissella halotolerans*	98
14	JX409628	Fermented whey permeate	This study	0.47	4.50	*L. plantarum*	96
15	JX409635	Whey permeate	This study	0.47	4.52	*L. plantarum*	98
17	NR_040815.1	Fermented sausage	(Collins et al., 1993)	0.45	4.4	*Weissella paramesenteroides*	99
19	JX409629	Piglet feces	This study	0.48	4.14	*Weissella viridescens*	99
21	JX409636	Piglet feces	This study	0.6	4.12	*L. casei*	100
23	JX409637	Piglet feces	This study	0.47	4.26	*L.plantarum*	100
26	JX409638	Fermented liquid feed	This study	0.23	4.87	*Pediococcus pentosaceus*	99
35	JX409639	Fermented liquid feed	This study	0.32	5.17	*Pediococcus pentosaceus*	99
37	JX409640	Fermented liquid feed	This study	0.41	4.50	*L. plantarum*	100
40	JX409641	Fermented liquid feed	This study	0.46	4.61	*L. plantarum*	99
43	JX409630	Piglet ileum	(Missotten et al., 2009)	0.47	4.82	*L. brevis*	99
46	JX409642	Fermented liquid feed	This study	0.44	4.58	*L. plantarum*	99
49	JX409643	Fermented liquid feed	This study	0.30	4.74	*L. plantarum*	99
52	JX409644	Fermented liquid feed	This study	0.34	4.87	*L. plantarum*	99
54	JX409645	Fermented liquid feed	This study	0.69	4.3	*L. plantarum*	100
57	JX409646	Fermented liquid feed	This study	0.61	4.73	*L. plantarum*	99
60	JX409647	Fermented liquid feed	This study	0.54	4.23	*L. kimchii*	99
65	JX409648	Fermented liquid feed	This study	0.73	4.25	*L. plantarum*	99
90	JX409649	Piglet feces	This study	0.53	4.20	*L. plantarum*	99
93	JX409650	Pig feed additive	This study	1.16	3.70	*L. rhamnosus*	100
94	JX409651	Pig feed additive	This study	1.36	3.97	*Enterococcus faecium*	99
95	JX409652	Pig feed additive	This study	1.15	3.78	*L. plantarum*	99
99	JX409653	Whey permeate	This study	1.27	3.67	*L. rhamnosus*	99
100	JX409631	Whey permeate	This study	1.25	3.63	*L. rhamnosus*	99
101	JX409632	Whey permeate	This study	1.36	3.74	*L. rhamnosus*	99
104	JX409633	Fermented liquid feed	This study	0.64	4.51	*L. plantarum*	100
106	JX409634	Fermented liquid feed	This study	0.75	4.25	*L.casei*	99
109	JX409627	Cheese	(Bernbom et al., 2006)	0.48	4.46	*L. plantarum*	100

*Initial pH of RWP was 6.00
§http://www.ncbi.nlm.nih.gov/BLAST/
§§ Designated accession number for NCBI GenBank library

Table 2 Antimicrobial activities of selected RWP fermenting LAB isolates (n = 3)*

Isolate (16S rRNA sequencing)	Inhibition zone (mm) of fermentation broth against indicated pathogens									
	MRS					7 % RWP				
	E. coli F4	*Strep. suis*	*L. monocytogenes*	*S.* Typhimurium	*Cl. perfringens*	*E. coli* F4	*Strep. suis*	*L. monocytogenes*	*S.* Typhimurium	*Cl. perfringens*
L. plantarum 15	3.3 ± 0.6	8.0 ± 1.0	3.7 ± 0.6	2.0 ± 0.0	10.0 ± 2.6	2.7 ± 2.3	3.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 0.7
W. paramesenteroides 17	4.3 ± 0.6	5.0 ± 2.6	3.7 ± 1.2	0.0 ± 0.0	10.0 ± 2.6	2.0 ± 1.7	3.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.0
W. viridescens 19	4.3 ± 0.6	5.7 ± 1.2	3.3 ± 0.6	0.0 ± 0.0	10.0 ± 3.6	1.0 ± 1.7	4.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.0
L. casei 21	3.0 ± 0.0	8.0 ± 0.0	4.0 ± 0.0	2.0 ± 1.7	4.7 ± 2.1	4.0 ± 0.0	5.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.0
L. plantarum 54	3.0 ± 0.0	8.7 ± 1.5	2.3 ± 0.6	0.0 ± 0.0	4.7 ± 0.6	1.3 ± 2.3	4.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L. plantarum 65	4.3 ± 0.6	6.3 ± 1.5	3.0 ± 0.0	1.7 ± 0.6	10.0 ± 3.5	2.7 ± 2.5	3.7 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L. rhamnosus 93	4.7 ± 0.6	6.3 ± 0.6	3.7 ± 1.2	1.0 ± 1.0	9.0 ± 4.6	4.3 ± 0.6	3.3 ± 0.6	3.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.0
Enterococcus faecium 94	3.0 ± 0.0	7.0 ± 1.7	7.7 ± 0.6	2.0 ± 0.0	11.0 ± 3.6	3.0 ± 0.0	4.0 ± 1.0	7.7 ± 0.6	3.0 ± 0.0	3.0 ± 0.0
L. plantarum 109	3.0 ± 0.0	7.3 ± 0.6	18.7 ± 1.5	2.7 ± 0.6	11.7 ± 1.5	0.0 ± 0.0	4.3 ± 0.6	12.7 ± 0.6	0.0 ± 0.0	4.0 ± 0.0

*Results are shown as mean ± S.D, Three independent assays were performed

Table 3 Survival of selected LAB isolates in simulated gastrointestinal conditions and their ability to attach to the epithelial cell line

Isolate	Gastric juice survival[*]	Bile Tolerance[†]	Cell adhesion score [±]
L. plantarum 15	6.23 ± 2.50^{bc}	2.0	5.0 (4.75-5.0)
W. paramesenteroides 17	42.5 ± 6.02^{a}	2.0	ND
W. viridescens 19	45.9 ± 0.29^{a}	2.0	ND
L. casei 21	1.99 ± 0.49^{cd}	2.0	ND
L. plantarum 54	3.14 ± 1.31^{cd}	2.0	5.0 (3.0-5.0)
L. plantarum 65	13.4 ± 3.65^{b}	2.0	5.0 (3.0-5.0)
L. rhamnosus 93	0^{d}	0.5	5.0 (2.0-5.0)
Enterococcus faecium 94	0^{d}	0	ND
L. plantarum 109	0^{d}	0.3	ND

ND: not determined

[*] Percentage survival of isolates after incubation in synthetic gastric juice for 30 min (pH 1.85).

[a,b,c,d]Different superscripts within the same column were different ($P < 0.05$)

[†]Values represented the maximum concentration of porcine bile (% w/v) at which growth was observed on MRS plates

[±]Values were expressed as median and interquartile range

Table 4 Growth of selected isolates, production of lactic acid and cell densities

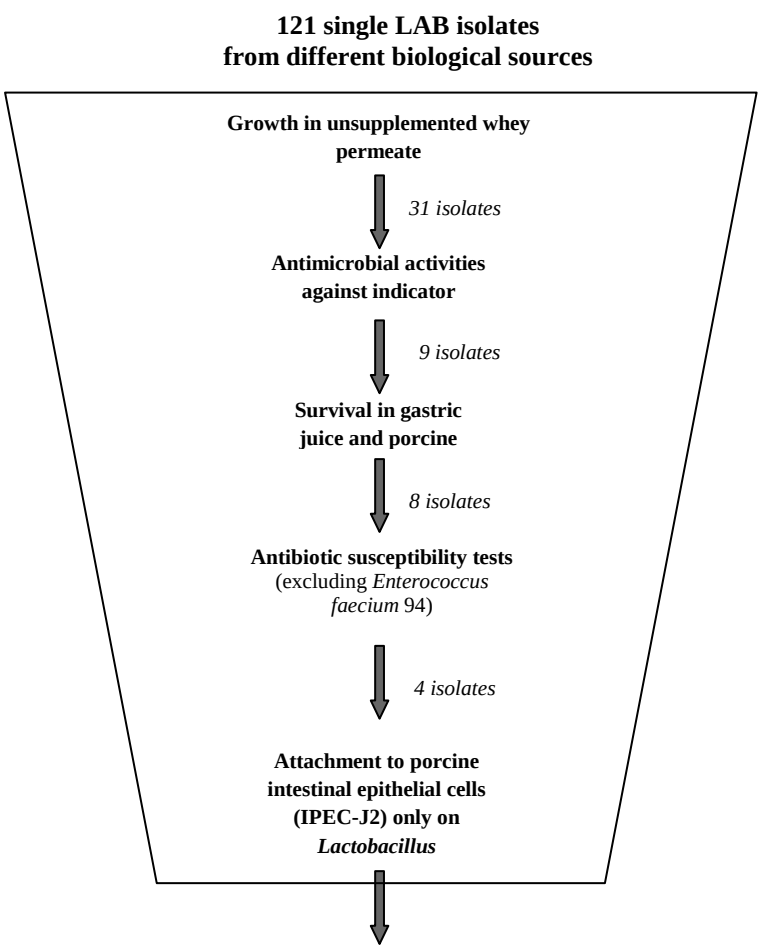
Isolate	Media	Temp (°C)	μmax (h^{-1})	Lactic acid (mmol kg^{-1}) after 24 h	Viable count (x 10^8 CFU mL^{-1})
L. plantarum 15	MRS	25	0.372	N/A	0.99
	MRS	37	0.454	231	
	RWP	25	0.010	N/A	
	RWP	37	0.030	8.70	
L. plantarum 54	MRS	25	0.388	N/A	1.4
	MRS	37	0.356	238	
	RWP	25	0.010	N/A	
	RWP	37	0.019	14.4	
L. plantarum 65	MRS	25	0.396	N/A	1.1
	MRS	37	0.357	219	
	RWP	25	0.018	N/A	
	RWP	37	0.022	15.3	
L. rhamnosus 93	MRS	25	0.200	N/A	11
	MRS	37	0.216	239	
	RWP	25	0.016	N/A	
	RWP	37	0.030	35.6	

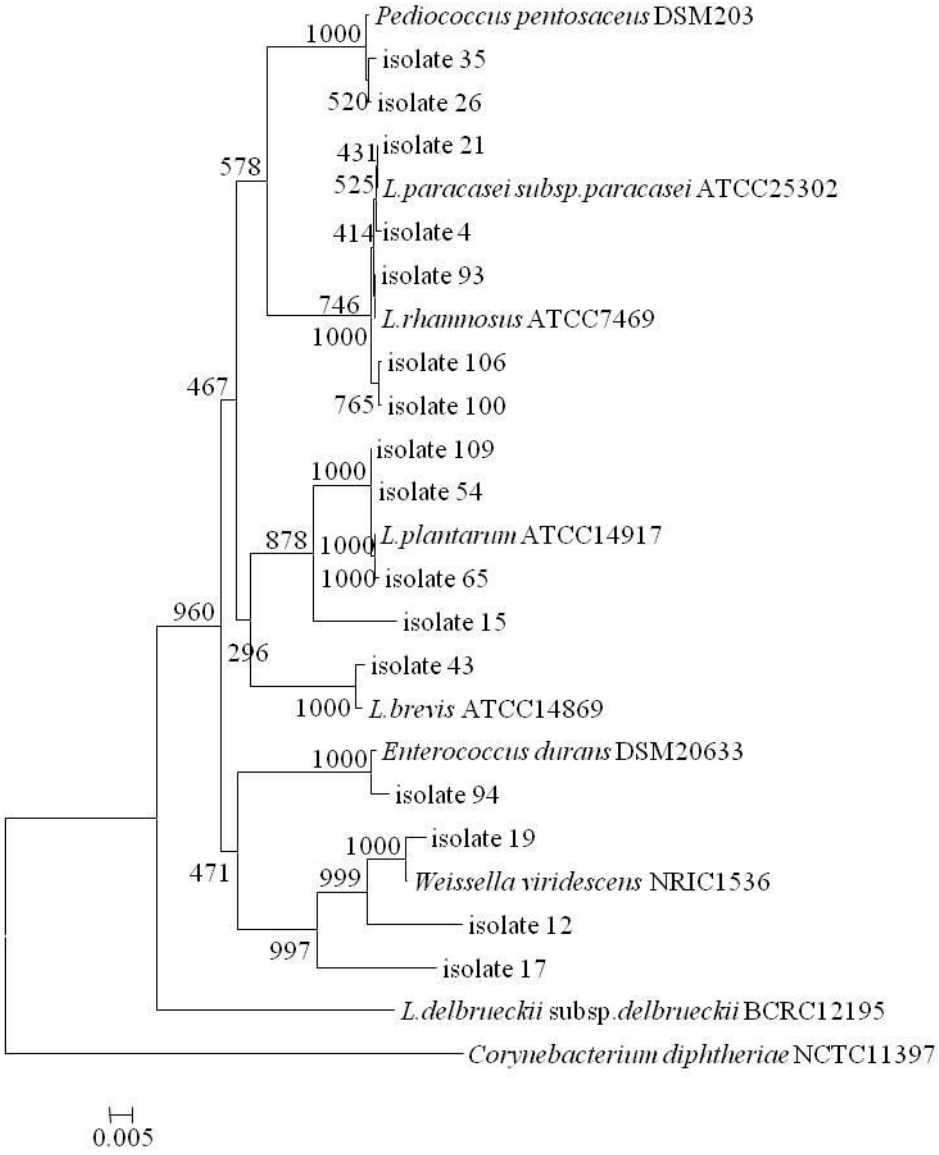
Figures

Fig. 1. Screening stages of lactic acid bacteria isolated from different natural sources

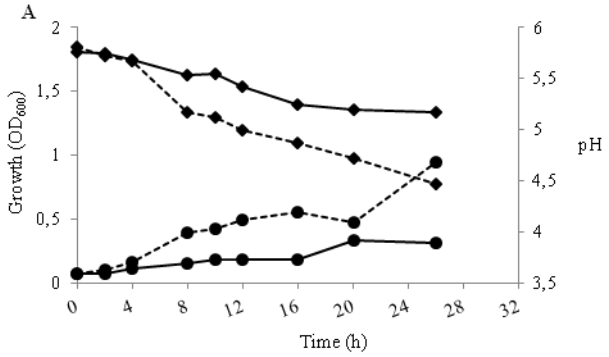
Fig. 2. Phylogenetic tree analysis of the 16S rRNA gene sequences of whey permeate growing isolates. The neighbor joining trees were built using the Clustal_X and MEGA4. Each number on a branch represents the bootstrap 1000.

Fig. 3. Growth curve and pH reduction of selected LAB isolates in RWP at 25^0 C (— solid line) and 37^0 C (- - - discontinued line). A. *L. plantarum* 15; B. *L. plantarum* 54; C. *L. plantarum* 65; D. *L. rhamnosus* 93.

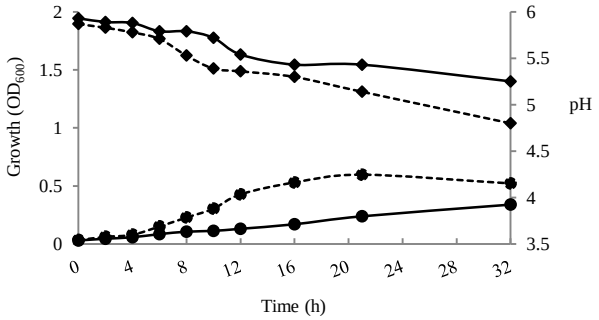

121 single LAB isolates
from different biological sources
Growth in unsupplemented whey permeate
31 isolates
Antimicrobial activities against indicator
9 isolates
Survival in gastric juice and porcine
8 isolates
Antibiotic susceptibility tests (excluding Enterococcus faecium 94)
4 isolates
Attachment to porcine intestinal epithelial cells (IPEC-J2) only on Lactobacillus
4 isolates


Pediococcus pentosaceus DSM203
1000
isolate 35
520
isolate 26
431
isolate 21
578
525
L.paracasei subsp.paracasei ATCC25302
414
isolate 4
isolate 93
746
L.rhamnosus ATCC7469
1000
isolate 106
467
765
isolate 100
isolate 109
1000
isolate 54
878
1000
L.plantarum ATCC14917
1000
isolate 65
960
isolate 15
296
isolate 43
1000
L.brevis ATCC14869
1000
Enterococcus durans DSM20633
isolate 94
1000
isolate 19
471
999
Weissella viridescens NRIC1536
isolate 12
997
isolate 17
L.delbrueckii subsp.*delbrueckii* BCRC12195
Corynebacterium diphtheriae NCTC11397
0.005

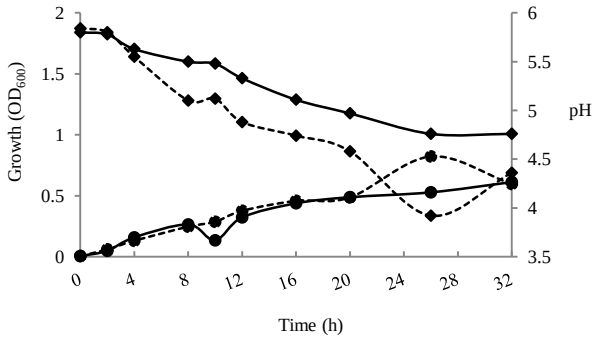
A


Growth (OD600)
pH
Time (h)

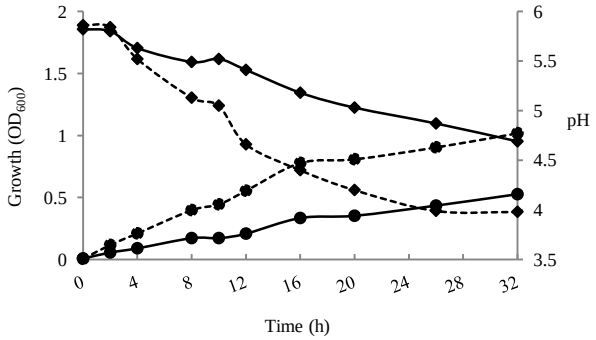
B


Growth (OD600)
pH
Time (h)

C


Growth (OD600)
pH
Time (h)

D


Growth (OD600)
pH
Time (h)

Supplementary Data

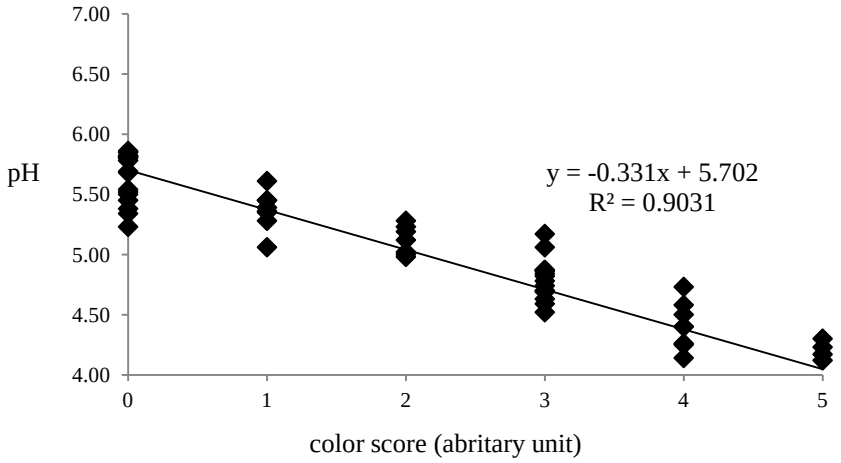


Fig. S1. Correlation between arbitrary units assigned to color changes resulted from growing LAB isolates in 7% RWP supplemented with Bromophenolblue.

Table S2 Identification and determination of antimicrobial activities of RWP fermenting LAB isolates after grown in MRS media.

Isolate	16S rRNA gene sequencing	MRS broth		Inhibition zone (mm) against indicated pathogen											
		pH	OD_{600}	*E. coli* F4			*Strep. suis*			*Listeria. monocytogenes*			*S.* Typhimurium		
				‡A	B	C	A	B	C	A	B	C	A	B	C
4	*L. casei*	4.31	3.47	0	0	0	2	5	3	0	0	0	3	3	4
10	*L. plantarum*	4.00	6.90	0	0	2	0	0	0	3	0	0	4	0	3
14	*L. plantarum*	3.97	7.19	2	0	0	5	4	0	0	0	0	3	3	3
15	*L. plantarum*	3.98	6.92	3	1	2	4	4	0	2	0	0	3	3	3
21	*L. casei*	3.95	6.46	3	3	3	5	5	0	2	0	2	0	3	3
23	*L. plantarum*	4.85	7.47	0	0	0	4	0	0	5	0	0	3	3	3
37	*L. plantarum*	4.99	9.85	0	0	0	0	0	0	4	0	0	4	3	3
40	*L. plantarum*	4.87	9.34	0	0	0	3	0	0	0	0	0	0	4	0
43	*L. brevis*	5.61	1.25	0	0	0	0	0	0	0	0	0	0	4	0
46	*L. plantarum*	4.86	9.21	3	0	0	4	0	0	0	0	0	0	0	0
49	*L. plantarum*	5.04	4.26	0	0	0	0	0	0	0	0	0	4	0	3
52	*L. plantarum*	5.10	8.42	0	0	0	0	0	0	3	0	0	4	4	3
54	*L. plantarum*	3.87	9.01	3	2	2	4	6	3	4	0	0	4	4	0
57	*L. plantarum*	4.01	3.40	0	0	0	5	4	0	3	0	0	0	3	0
60	*L. kimchii*	4.31	2.75	0	0	0	5	4	0	0	0	0	0	0	0
65	*L. plantarum*	3.87	10.39	3	0	0	6	3	4	2	0	0	0	3	0
90	*L. plantarum*	4.05	6.59	0	0	0	0	0	0	0	0	0	0	0	0
93	*L. rhamnosus*	3.85	9.13	4	2	2	5	4	0	4	0	0	3	3	0
95	*L. plantarum*	3.90	8.49	0	0	0	3	3	0	0	0	0	0	0	0
99	*L. rhamnosus*	3.97	5.26	0	0	0	3	3	0	0	0	0	0	0	0
100	*L. rhamnosus*	3.97	6.00	0	0	0	3	0	0	0	0	0	0	0	0
101	*L. rhamnosus*	3.98	6.12	0	0	0	0	0	0	0	0	0	0	0	0
104	*L. plantarum*	4.81	9.12	0	0	0	0	0	0	0	0	0	0	0	0
106	*L. casei*	4.75	7.86	0	0	0	0	0	0	0	0	0	0	0	0
109	*L. plantarum*	4.63	7.81	3	3	0	4	3	0	17	13	13	0	0	0
12	*W. halotolerans*	4.42	3.27	0	0	0	4	3	0	0	0	0	0	0	0
17	*W. paramesenteroides*	3.96	6.5	3	1	2	4	4	0	3	0	0	3	3	3
19	*W. viridescens*	3.94	7	3	2	2	5	3	3	3	0	0	3	3	4
26	*Pediococcus pentosaceus*	5.08	5.3	0	0	0	3	0	0	5	0	0	3	0	3
35	*P. pentosaceus*	5.13	7.01	0	0	0	0	0	0	4	0	0	3	3	3
94	*Enterococcus faecium*	3.96	7	3	0	0	0	0	0	4	3	4	0	0	0

‡ Cultures were fractioned into: A – whole culture broth; B – neutralized resuspended cells; C – supernatant

Table S3 Minimum Inhibitory Concentrations (MICs) against antibiotics of selected LAB isolates.

Isolate	MIC (μg mL^{-1})																						
	AMP[a]	VAN[a]	GEN[a]	KAN[a]	STR[a]	ERY[a]	CLI[a]	SYN[a]	TET[a]	CHL[a]	PEN	SMX	SPE	NAL	NEO	TMP	CIP	COL	FFN	SAL	AUG	APR	FOT	XNL
L. plantarum 15	2	32	0.5	< 8	8	0.12	0.12	2	32	4	8	64	16	64	2	1	4	16	2	2	2	4	4	8
W. paramesenteroides 17	1	32	0.5	8	8	0.12	0.12	2	***16***[b]	2	8	64	16	64	2	1	4	8	2	2	2	4	4	8
W. viridescens 19	1	32	0.5	8	8	0.12	0.12	2	***8***[b]	2	4	64	16	64	2	1	4	8	2	2	2	4	4	8
L. casei 21	1	32	0.5	8	8	0.12	0.12	2	***16***[b]	2	4	64	16	64	2	1	4	16	1	2	2	4	4	8
L. plantarum 54	1	32	0.5	8	8	0.12	0.12	2	16	4	4	64	16	64	2	1	4	16	2	2	2	4	2	8
L. plantarum 65	2	32	0.5	8	8	0.12	0.12	2	8	2	1	1024	16	64	2	1	4	4	2	2	2	4	1	8
L. rhamnosus 93	1	32	0.5	8	8	0.12	0.12	1	2	2	0.5	1024	16	64	2	1	0.5	32	2	2	2	8	4	8
L. plantarum 109	4	32	0.5	8	8	0.12	0.12	1	8	2	2	64	16	64	2	1	4	8	2	2	2	4	4	8

AMP: ampicillin, VAN: vancomycin, GEN: gentamycin, KAN: kanamycin, STR: streptomycin, ERY: erythromycin, CLI: clindamycin, SYN: synercid (a trade name for quinupristin + dalfopristin, TET: tetracyline, CHL: chloramphenicol, PEN: penicillin, SMX: sulphamethoxazole, SPE:Spectinomycin, NAL: naladixan, NEO: neomycin, TMP: trimethoprim, CIP: ciprofloxacin, COL: colistin, FFN: florfenicol

[a] Antibiotics that are included in the assessment of bacterial resistance to antibiotics of human or veterinary importance

[b] MIC values are higher than EFSA recommended breakpoints for the specific species

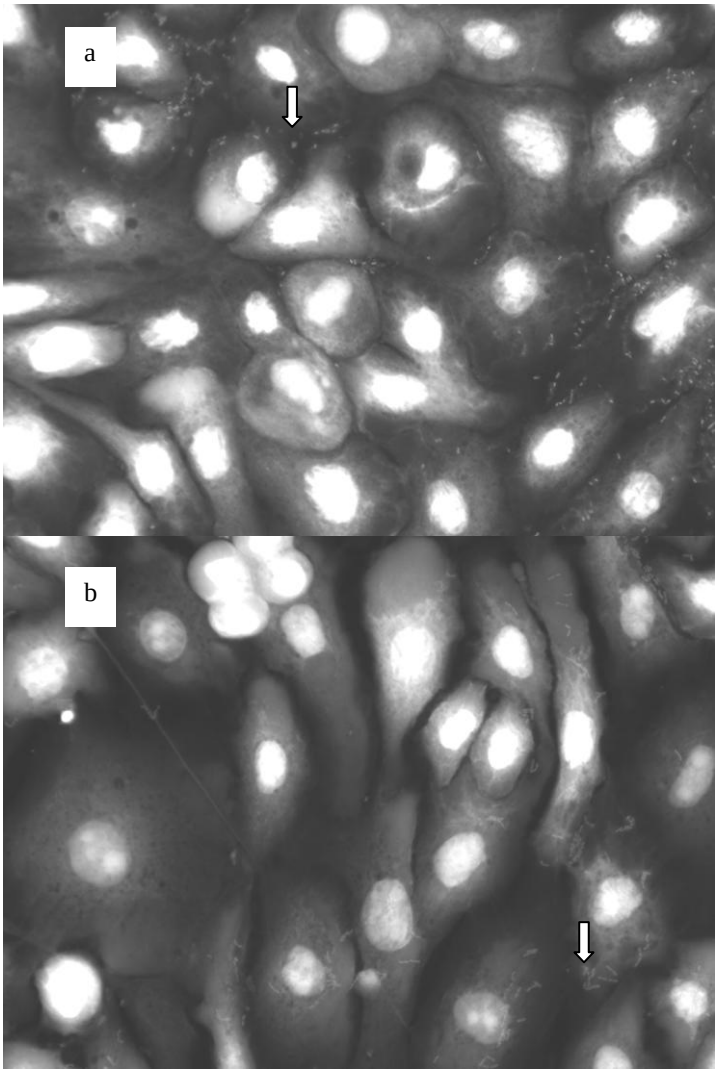


Fig. S4. Attachment of selected isolates (a) *L. plantarum* isolate 54 and (b) *L. plantarum* isolate 65 on IPEC-J2 cell line. Experimental conditions were explained in the Materials and Method. Microscope: 400x magnification

Paper 3. Early draft

Whey permeate fermented with *Weissella viridescens* reduced diarrhoea, modulated the intestinal microenvironment and gastrointestinal microbiota of post weaned piglets challenged with *Escherichia coli* F4

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Running title: Fermented whey permeate reduced *E. coli* diarhoea in post weaned piglets

Keywords: *Weissella viridescens*, whey permeate, post weaning, piglets, diarrhoea, microbiota

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Abstract

Addition of probiotic in the feed reportedly alleviates diarrhoea symptoms among post weaned piglets. Whey permeate (WP) is an abundant source of lactose which has shown to improve growth performance in growing pigs (Pierce et al., 2007)The objective of the present study is to evaluate the addition of fermented whey permeate inoculated with selected lactic acid bacteria, in reducing diarrhoea symptoms among piglets challenged with enterotoxigenic (ETEC) *Escherichia coli* F4. The experiment involved 52 piglets from 5 litters, including a control group (non-challenged), and 5 challenged groups covering a control group without WP, a group fed with non- fermented WP, and 3 groups receiving WP fermented with different potential probiotic inoculums (a) *Lactobacillus plantarum*, (b) *Streptococcus thermophilus*/*Lactobacillus bulgaricus*, and (c) *Weissella viridescens*, respectively. During the entire trial, the animals' health status, body weight, and microbial parameters were monitored . Ileum and colon microbiota communities were analysed using Illumina Hiseq based on 16S rRNA gene sequencing of the V1 hypervariable region. Addition of whey permeate, with or without lactic acid bacteria inoculums, reduced the number of days with diarrhoea. *Weissella viridescens* inoculations of whey permeate reduced diarrhoea frequency, improved feed intake, increased production of butyric acid and abundance of the phylum Firmicutes in colon. The experiment showed that adding *W. viridescens* as inoculum to whey permeate might be an economical yet efficient practice to prepare probiotic feeding to post weaned piglets.

Introduction

The weaning period in pigs is often accompanied by transient diarrhoea responsible for increasing morbidity and mortality (Lalles et al., 2007). Enterotoxigenic *Esherichia coli* F4 is frequently linked to postweaned diarrhoea globally (Frydendahl et al., 2003; Konstantinov et al., 2008; Krause et al., 2010; Zhang et al., 2010). On the other hand, one of the most documented health effects of probiotics is the reduction of diarrhoea in children/infant (Szajewska and Mrukowicz, 2001; Johnson et al., 2007; Guandalini. 2008). Accordingly, feeding probiotics has been proposed as an alternative to antibiotic as preventive and curative treatment to *E. coli* F4 infections (Daudelin et al., 2011; Trevisi et al., 2011)(Konstantinov et al., 2008).

To be able to evaluate the modulating effects of probiotic, piglet infection models becomes crucial. The main challenge is to obtain *E. coli* F4 infections which generate observable

clinical symptoms without abrupt mortality to allow enough post- infection period to observe changes due to probiotic administration. For practical relevance, it is also desirable that the infection model closely reproduces farm-like conditions (Schroeder et al., 2006). Establishing susceptibility to *E. coli* F4 colonization in piglet provides desirable experimental ETEC-induced diarrhoea (Jensen et al., 2006).

Administrations of different strains of probiotics of various natural origins have provided some promising results on challenged piglets in the last 10 years. These include elevation of diarrhoea symptoms soon after infection (Krause et al., 2010; Zhang et al., 2010), reduction of *E.coli* F4 attachments to the intestinal mucosa (Daudelin et al., 2011), and reduction of *E. coli* F4 in ileum contents (Konstantinov et al., 2008). Furthermore, probiotics help in the regulation of systemic inflammatory cytokines (IL-6, TNF-α) (Zhang et al., 2010; Daudelin et al., 2011). Lastly, there was an improved average daily gain especially when probiotic was added together with fermentable carbohydrate (Bhandari et al., 2009; Krause et al., 2010). However, less encouraging results in which challenged piglets health status became worse after addition of *L. rhamonsus* GG has also been reported (Trevisi et al., 2011).

Lactose is a digestable disaccharide which for years have been added to the feed especially to improve feed intake among post weaned piglets (Bertol et al., 2000; Cromwell et al., 2008). Whey permeate is a by-product from cheese processing and contains an abundant amount of lactose. It is considered an excellent medium for growing lactic acid bacteria (Paper 2, Hugenschmidt et al., 2010; Naranjo et al., 2010). Previously, our group has screened and characterized 5 lactic acid bacteria that grew in whey permeate and exhibit probiotic properties (Paper 2).

The current study incorporated whey permeate, which has been fermented by three different lactic acid bacteria inoculums as economical probiotic preparation, to *E. coli* F4 challenged post weaned piglets. We aimed to evaluate the efficacy of this practice in reducing diarrhoea symptoms, improving growth performance, and modulating gut microbial communities.

Materials and Methods

Animals

The piglets in this study were obtained from the herd at the Research Center Foulum, University of Aarhus. The herd has the specific-pathogen-free (SPF) health status according to the Danish SPF system (i.e. free from toxigenic *Pasteurella multocida*, *Sarcoptes scabei* var. *suis*, *Haemotopinus suis*, *Brachyspira hyodysenteriae*, and *Actinobacillus pleuropneumoniae* serotype 1,2,3,4,5,7,8,9,10, but reinfected with *Mycoplasma hyopneumoniae*). Only sows tested homozygote carriers of the dominant gene encoding for intestinal F4 fimbriae receptors (Joergensen et al., 2004) were used as dams. Regardless of genotype, the density of intestinal receptors for *E. coli* F4 adhesion is variable (Rasschaert et al., 2007). A total of 52 piglets were obtained from 5 litters over a period of 6 months. From each litter, between 8 to 12 piglets were weaned at 30 ± 2 days of age and assigned for experimental diets. The animal experiment was conducted at Department of Animal Science, Research Center Foulum, Univesity of Aarhus, Denmark. The procedure was approved by the Danish Ethical Commission with respect to animal experimentation and care of animals under study enforced by the Commission.

Preparation of fermented whey permeate

Whey permeate was fermented using three different inoculums. The two inoculums included lactic acid bacteria (LAB) strains, *Lactobacillus plantarum* and *Weissella viridescens*, which were previously isolated and characterised (Paper 2) and the third inoculum contained *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The preparations of fermented whey permeate using *L. plantarum* or *W. viridescens* are shown in Figure 1. The separate inoculums were prepared as follows: a loop of frozen -80 °C respective pure isolate was streaked on MRS agar plate and incubated at 37 °C for 18 h under aerobic condition. A single colony from the plate was picked for inoculation of MRS broth. The tube containing inoculated MRS broth was incubated at 37 °C for 17 h under aerobic condition. Four ml of this overnight suspension were added to 1 L reconstituted whey permeate (RWP 70 g Variolac® to 930 ml tap water) which has previously been incubated at 37 °C for 18 h followed by pasteurization and addition of 1 g/L yeast extract. The pH was adjusted to 6.0 with 0.4 M KOH prior to addition of 4 ml overnight inoculum suspension. The third fermented whey permeate was prepared by adding a commercial product containing *S. thermophilus* and *L. bulgaricus* (ChrHansen A/S, Denmark). In brief, 2 g of the commercial

cell paste were added to 1 L of 7% (m/v) RWP supplemented with 1 g/L yeast extract followed by incubation at 37 °C for 17 h. All 3 fermented whey permeate were kept at 4 °C until use. Samples were taken from representative fermentations (N=4) for measurement of pH and enumeration of lactic acid bacteria, enterobacteria and yeast. Enumeration of the microorganisms was done as described for the faecal samples. The fermented WP was mixed with the basal diet (Table 1) to reach a final concentration of 6.4% WP. For the control diet (without WP addition), feed and water were mixed in the ratio 1:2.5. For WP groups, the feed to water to non-fermented or fermented WP ratio were adjusted to 1:1.6:0.9 (Table 2).

Experimental design

Five separate experiments based on litter were performed to evaluate the response from piglets to treatments. The 6 treatment groups were: (1) no *E. coli* F4 challenge no WP (No F4), (2) *E. coli* F4 challenge no WP (F4), (3) *E. coli* F4 challenge fed with 64 g/kg non-fermented WP (F4+WP), (4) *E. coli* F4 challenge fed with 64 g/kg WP fermented with *S. thermophilus*/*L. bulgaricus* (F4+WP+Pro1); (5) *E. coli* F4 challenge with 64 g/kg WP fermented with *L. plantarum* (F4+WP+Pro2) and (6) *E. coli* F4 challenge fed with 64 g/kg fermented with *W. viridescens* (F4+WP+Pro3).

There were variations in the number of piglets available from each litter which resulted in an uneven number of individuals in each treatment group. At the time of writing this article, 6 piglets were included in No F4 group, 8 piglets in F4 group, 10 piglets in F4+WP group, 8 piglets in F4+WP+Pro1 group, 10 piglets in F4+WP+Pro2 and F4+WP+Pro3 groups. The responses from the available piglets from these experiments were primarily used to choose the best LAB to prepare fermented whey permeate. Two additional experiments were planned in January and February 2013 to provide a greater number of piglets receiving fermented whey permeate by the selected LAB.

Housing and feeding

Eight to ten piglets from each litter were weaned at 30 ± 3 days and a body weight (BW) of 7.84 ± 0.05 kg prior to being transported to pens (184 x 82 cm, of which 82 x 82 was slatted) two by two. Each pair was allotted to one of the six treatments. No physical contact between piglets from different pens was allowed. Piglets were fed with dry basal diet with the addition of water and whey permeate (Table 1). Inclusion of whey permeate was compensated with wheat level in the experimental diets. The amount of dehulled toasted

soybean meal and the addition of the synthetic amino acids lysine, methionine, threonine and tryptophan were adjusted to optimize the diet with regards to protein and amino acid composition (Table 1). The animals were fed the experimental diets two times per day at 8.00 a.m. and afternoon 15.00 p.m., respectively throughout the study and given free access to water. The amount of feed given each day is shown in Table 3. Residual feed were removed and registered every morning. The pigs were given free acces to water throughout the study. Feed intake and body weight were registered on Day 1, 4, 9, and 11.

Challenged with E. coli F4

Escherichia coli 9910045-1:0149ST2,LT,F4ac (hereafter is written as *E. coli* F4), was first isolated at the Danish Veterinary Insitute (Frydendahl et al., 2001). *E. coli* F4 was stored at –80 °C in Luria Bertani (LB) medium (Merck) with 20% (m/v) glycerol. For each inoculation, a fresh culture was prepared. Frozen *E. coli* F4 was streaked on Blood Agar (BA) plate and grown at 37 °C for 18 h. A single colony was swabbed and suspended in 200 ml Veal infusion broth (Merck) and incubated for 5 h at 37 °C in an incubator with constant shaking at 200 rpm. *E. coli* F4 cells were collected by centrifugation (12,000 x g) at 4 °C for 20 min. The pellet was resuspended in sterile 0.9% sodium chloride (NaCl). This bacterial suspension was diluted in serial ten-fold dilutions with NaCl as diluent and was plated on BA for enumeration of *E. coli* F4. On Day 2 and Day 3 after morning meal, each piglet was challenged with *E. coli* F4 5 x 10^7 in 20 ml 0.9% NaCl via an oro-gastric tube. Non challenged piglets received equivalent amounts of sodium bicarbonate via an oro-gastric tube.

Faecal score

Faecal score (1 = hard and dry; 2 = firm; 3 = soft, but able to retain some shape; 4 = soft and unable to retain any shape; 5 = watery and dark; 6 = watery and yellow; 7 = foamy and yellow) were evaluated by visual appraisal on day 2,3,4,5,6 and 8 by a technician who was blinded from the treatment groups. Piglet was categorized as having diarrhoea symptom with a faecal score of 4 or higher.

Faecal sampling and analyses

Faecal samples were taken from the rectum of piglets on day 2,3,4,5,and 9 of the experiment. Sampling on day 2 was conducted before infection with *E. coli* F4. Faeces were analysed for dry matter (DM) content (AOAC, 1980) and for microbiology counts (coliform bacteria, hemolytic *E. coli*, lactic acid bacteria, and total anaerobic bacteria). Approximately 1 g of faeces samples were transferred rapidly after collection under a flow of CO_2 into a CO_2-

flushed plastic bag and diluted 10 times with a pre-reduced salt medium (Holdeman et al., 1977) and homogenized in a stomacher blender (Interscience, St. Nom, France) for 2 min. Then, 10-fold dilutions were prepared in peptone water for the feed samples and in pre-reduced salt medium for the digesta samples as previously described (MILLER and WOLIN, 1974). Samples (0.1 ml) were plated on non selective and selective media. Total anaerobic bacteria in digesta samples were enumerated by culturing the samples in roll tubes containing pig colon fluid-glucose-cellobiose agar (Holdeman et al., 1977) and incubating anaerobically at 37 ± 1 ºC for 7 days. Lactic acid bacteria were determined on de Man, Rogosa, and Sharpe (MRS) agar (Merck) after anaerobic incubation at 30 ± 1 ºC for 3 days for feed samples or anaerobic incubation at 37 ºC for 2 days for digesta samples, respectively. Enterobacteriaceae including coliforms in feed and digesta samples were enumerated on McConkey agar (Merck) after aerobic incubation at 37 ºC for 1 day. Haemolytic *E. coli* were enumerated on BA after aerobic incubation at 37 ± 1 ºC for 1 day. Growth of colonies with Yeasts and molds were enumerated on malt chloramphenicol agar (MCA) [10 g/l of glucose (Merck); 3 g/l of malt extract (Merck); 3 g/l of yeast extract (Merck); 5 g/l of Bacto peptone (Merck); 50 mg/l of chloramphenicol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); and 15 g/l of agar (Merck)] following aerobic incubation at 30 ºC for 3 days for feed samples and aerobic incubation at 37 ºC for 2 days for digesta samples.

End of experiment

On day 11 of the experiment at a BW of 11.7 ± 3.4 kg, piglets were sacrificed by a captive bolt gun 3 h after morning meal. The gastrointestinal tract (GIT) was immediately removed, measured and divided into 8 segments: stomach, 3 equal (length) parts of the small intestines, caecum, and 3 equal (length) parts of the colon including the rectum. The total contents of each segment were weighed and within 5 min, the pH was determined. Digesta from stomach, the distal segment of small intestines, caecum, and colon were immediately analysed for microbial contents. Residual digesta samples from 8 segments of the GIT were stored at -20 ºC for analyses of dry matter (DM) and organic acids. Digesta from ileum and colon were collected and stored at -20 ºC until processing for DNA extractions. Gut tissue biopsies from stomach, small intestines, caecum, and colon were stored in formalin for morphological measurements.

Analytical methods

Dry matter content from the digesta was determined by freeze-drying the samples. The results of chemical analyses of the diets were expressed in DM percentage. DM was determined by drying the samples at 103 ºC until constant weight was reached (European Union, 1971). The concentration of SCFA, lactic acid, and succinic acid were measured as previously described (Canibe and Jensen, 2007).

Microbial determinations

Approximately 10 g of digesta samples were transferred rapidly after collection under a flow of CO_2 into to a CO_2-flushed plastic bag and diluted 10 times with a pre-reduced salt medium (Holdeman et al., 1977) and homogenized in a stomacher blender (Interscience, St. Nom, France) for 2 min. Microbial enumeration was performed as described for faecal samples.

Histomorphometry

Measurement of the total thickness of mucosa (villous height and crypt depth combined) was done under blind conditions as previously described (Marion et al., 2005) with slight modifications. Cross sections of different parts of the GIT tract (stomach, duodenum, jejunum, ileum, and colon) were obtained and processed as routinely for histology. Serial sections of 3 µm thickness were stained with hematoxylin-eosin. An overview observation across 5 GIT tract sections was performed. Furthermore, 10 random determinations of total thickness of mucosa were performed for jejunum and colon samples. Measurements were performed using a Zeiss optical binocular microscope under low magnification power (2.5 x objective lens) coupled with Axio camera to the PC computer supported by the AxioVision rel 4.8 software (Carl Zeiss GmbH, Jena, Germany).

DNA extraction

DNA was extracted from 200 mg digesta samples from either ileum or colon. Prior to extraction, to ensure complete lysis of bacteria, samples in TE buffer were incubated with lysozyme (25 mg ml^{-1}) for 2 h followed by centrifugation at 10,000 x g for 2 min. Supernatant was beaten in mini bead beater (Biospec, Bartlesville, OK) at 20 Hz for 2 min. Intracellular materials were collected in the supernatant after centrifugation at 10,000 x g for 2 min. DNA was extracted using a Promega LEV blood kit (Promega Biotech, Mannheim, Germany) according to the manufacture's instructions. Collected cells prior to extraction. representing the colonic microbial communities was extracted from individual colonic digesta samples using the QIASymphony virus/bacteria mini kit (Qiagen, Mainz, Germany)

according to the manufacturer's instructions. Purity of the extracted DNA was determined using UV absorption spectrums including OD 260/280 ratio on a Nanophotometer (Implen, Munich, Germany).

Polymerase chain reaction amplicon construction and sequencing

The PCR reactions were performed as previously described (Paper 1). The quality of the product was evaluated in a Bioanalyzer 2100 (Agilent, CA, USA) using the DNA 1000 LabChip (Agilent, CA, USA). Only PCR products without contaminant bands were used for sequencing. The PCR products from different pigs were pooled in equimolar ratios based on Nanophotometer (Implen, Munich, Germany) readings prior to phenol/chloroform precipitations. Products were eluted in eluent buffer and submitted for sequencing, including base calling, at the University of Copenhagen Sequencing Center at Copenhagen University, Denmark, for sequencing on an Illumina HiSeq^{TM} 2000 platform.

Sequencing data-analysis

Obtained data were analysed using the BION-meta software (ftp://genomics.dk/pub/BION, Larsen *et al.*, in prep). In brief, the sequences were initially de-multiplexed based on the primer- and barcode sequences. Sequences were cleaned at both ends by removal of bases of a quality less than 96%. A setting of 99.8% base quality was used to cluster and aligned identical sequences to obtain consensus sequences. Consensus sequences of at least 30 nucleotides in length, based on respective barcode, were mapped into a table. Phylogeny classification was performed by comparing consensus sequences against the Greengenes SSU database (http://greengenes.lbl.gov) using a word length of 8 and a match minimum of 70%. The top one percent of the obtained hits from the Greengene database was then used for taxonomical classification of the consensus sequences. The number of reads within each barcode was normalised to an arbitrary number (100,000) in order to calculate the relative abundance of a given bacteria between individuals in the experiment statistically.

Statistical analysis

Faeces samples from 5 piglets that died during the experiments were included in analyses of faecal score, % DM , hemolytic coliform and lactic acid bacteria counts.

The frequency of diarrhoea was determined by modeling diarrhoea, defined as faecal score of 4 or higher, with a logistic regression model with random effects, using the glmmPQL routine from the R computer package for the analysis. Fixed effects were Treatment, Day and dry matter, and the individual Pig was considered a random effect. Day being treated as a fixed

effect was due to the relatively large number of pigs which resulted in computational problems when including both Pig and Day as random effects. Results were thus corrected for effects of the individual day and the level of dry matter.

Microbiological counts from faecal samples were analysed using a 2-way ANOVA analysis with Bonferroni's post test in GraphPad Prism version 5.00 for Windows(GraphPad Software, San Diego California USA).

SCFA and CFU data were analysed by a mixed model including treatment, GIT segment, and treatment by GIT segment interaction using 2-way ANOVA in GraphPad Prism. The growth performance (ADG, ADFI) data were analysed using one-way ANOVA from the same software. When there was an overall significant effect on either diet at $P < 0.05$, Bonferroni's t-test was performed to compare means.

The effect of diet on ileal and colonic microbiota and morphometry was performed separately, using a non-parametric one-way ANOVA (Kruskal-Wallis). When there was an overall effect of diet on each taxonomic group, at $P < 0.05$, differences between means were compared pairwise using Dunn t-test.

Results

A total of 4 piglets, 1 piglet from F4 group and 3 piglets from the F4+WP+Pro1 group died during the experiment. Prior to death, piglets showed severe diarrhoea and lost of appetite. Abductions of GIT from deceased piglets were performed to determine the cause of mortality. Based on observations that there were watery digesta in the gastrointestinal contents, diarrhoea was determined as the common cause of death.

Feed chemical composition is provided in Table 2. The pH of fermented WP with *L. plantarum* or *W. viridescens* were 4.12 and 4.14, respectively. Each contained an average of 2.0×10^8 CFU/ml lactic acid bacteria. The pH of fermented WP with *S. thermophilus*/*L. bulgaricus* was 3.85 and contained approx. 2.0×10^7 CFU/ml lactic acid bacteria. There were less than 1000 yeast or coliform bacteria in all fermented WP.

Growth performance and gut morphology

The initial BWs of the piglets did not differ ($P > 0.05$) among dietary treatments (data not shown). The effect of diet treatments on growth performance is presented in Table 4. There were no statistical differences in average daily gains (ADG). However, the trend within the

first 4 days showed that F4 group grew the least while WP+Pro3 group gained the most weight. At the end of the experiment (Day 11), F4+WP group showed the most growth followed by F4+WP+Pro3. Fermenting WP with *L. plantarum* (F4+WP+Pro2) or *W. viridescens* (F4+WP+Pro3) resulted in significantly higher Average Daily Feed Intake (ADFI) ($P < 0.05$) than using *S. termophilus*/*L. bulgaricus* (F4+WP+Pro2).

Piglets number 33 and 34, which were part of the F4 group, showed evident villi atrophy in the jejunum and ileum, respectively. However, overall, the gut morphology of jejenum and colon was not affected by diet (Figure 2).

Diarrhoea incidence

Piglets were challenged with *E. coli* F4 at Day 2 and Day 3 of the infection trial. All groups exhibited diarrhoea symptom at Day 4 (Figure 3), indicated by median fecal score of 4 or higher. The number of days in which piglets showed diarrhoea symptom varied among treatment groups. No F4 group suffered from diarrhoea for 3 days, F4 group for 4 days, F4+WP group for 3 days, F4+WP+Pro1 and F4+WP+Pro2 for 3 days. The F4+WP+Pro3 group suffered the least period (2 days) of diarrhoea.

Addition of WP or fermented WP affected diarrhoea incidence. F4 group suffered from higher diarrhoea frequency ($P = 0.0318$) compared to the other groups. Albeit statistically insignificant there was a tendency of lower diarrhoea frequency when piglets were fed with fermented WP inoculated with *W. viridescens* ($P = 0.0928$).

We observed a reverse trend of faecal score and % DM (Figure 2b). On Day 3, which was the first day after the start challenge, faecal score started to increase (except for No F4 group). On Day 4, median faecal score for all groups reached 4.0 or above and corresponded to low mean value of % DM. On Day 6, when piglets fed with fermented WP inoculated with *W. viridescens* no longer exhibited diarrhoea symptom (median faecal score = 3.2), solid content in the faecal improved to 19%.

Organic acids and pH in the gut

Diet treatment affected the amount of organic acids and pH in the gut (Table 5). In caecum, propionic acid was higher in F4+WP and in F4+WP+Pro1 groups than in the No F4 (control) group ($P = 0.019$). Valeric acid, isobutyric acid, and isovaleric acid were also highest in the F4+WP+Pro1 group ($P = 0.034$). In colon, acetic acid ($P = 0.0026$) and butyric acid ($P =$

0.032) were highest in the F4+WP+Pro1 and F4+WP+Pro3 groups, while propionic acid was highest in F4+WP+Pro3 group (P = 0.019).

The acidity in the ileum was affected more by infection than by the addition of WP, either by itself, or after fermentation. pH was lower in the F4 group than in the No F4 group (P = 0.026).

Microbiology of faecal and digesta

Dietary treatments did not affect counts of hemolytic coliforms, lactic acid bacteria (Figure 3c and 4d), yeast or total anaerobic (data not shown) in faecal samples taken on different days during the experiment. Noted that starting on Day 4, there was an increased counts of hemolytic coliform in the non- challenged (No F4) group. This indicated a potential cross contamination during the experiment.

Diet treatments in general did not affect microbiology counts in the GIT digesta samples (Table 6). However, adding WP with or without fermentation seemed to increase the number of LAB in the ileum digesta (P = 0.059)

Gut bacterial community structure and diversity

Next generation sequencing on 16S rRNA gene amplicons was performed on an Illumina platform to provide an overview of microbial community structure in the ileum and colon digesta. In ileum, 7 most abundant phyla were identified, with the phylum Firmicutes being the most abundant. In colon, 8 most abundant phyla were found including Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Tenericutes, Proteobacteria, TM7, Spitochatest (Figure 4).

Diet treatments affected relative abundance of the phylum Chloroflexi in ileum (P = 0.048). Challenged piglets fed with fermented whey inoculated with *L. plantarum* (F4+WP+Pro2) consisted of more Chloroflexi than ileum of the non-challenged (No F4) group. *Lactobacillus* was the most abundant genus in the ileum. However, there were no statistical differences due to treatments.

Meanwhile in the colon, at phylum level, relative abundance of Firmicutes was higher in challenged piglets fed with *W. viridescens* (F4+WP+Pro3) than in the control (No F4) group. At genus level, *Blautia* was found most abundant, but again, diet treatment did not affect the abundance of genera in colon digesta.

Bacterial diversity in the digesta of ileum or colon were not affected by diet treatments (Figure 5). However, there was a trend towards less diversity in the ileum digesta than in the colon.

Discussion

The study investigated the effect of including whey permeate (WP) and fermented whey permeate prepared by different lactic acid bacteria on diarrhoea incidences, growth performance and community structure of gut microbiota in *E. coli*- challenged post weaned piglets. The numerical differences among treatments were relatively large, suggesting the modest number of replicates and variability of responses to *E. coli* infection. Similar observation was reported (Sorensen et al., 2009) when the authors observed inconsistent diarrhoea symptoms.

However, our observations in general indicated that the infection model was successful. There was a higher diarrhoea frequency in the F4 group than in other groups. Furthermore, we observed that adding fermented whey prepared by *W. viridescens* inoculum reduced frequency of diarrhoea. *W. viridescens* was originally isolated from pig's faeces and was characterized as being able to proliferate in whey permeate without supplementation. Furthermore, the strain exhibited potential as probiotic by inhibit *E. coli* F4 and survived in the presence of 2% (m/v) porcine bile salt (Paper 2).

Weaning is a stressful phase for piglets and they often times suffer of low feed intake (McCracken et al., 1999; Lalles et al., 2007). Piglets which maintained their appetite posses greater chance to recover from diarrhoea. Indeed, our study revealed that piglets in the F4+WP+Pro3 group which showed highest feed intake were the ones with less diarrhoea frequency. Interestingly, addition of non-fermented WP also improved feed intake, but no apparent reduction of diarrhoea frequency was observed. These results suggest that beyond the effect of WP, viable lactic acid bacteria, in this case, *W. viridescens* and the metabolites resulted from WP fermentation, contributed to the lowering of diarrhoea incidences in challenged piglets.

Our study observed that differences in diarrhoea frequencies were not explained by shedding of haemolytic coliform in faeces nor by changes in Enterobacteriaceae abundance in ileum digesta. To confirm the presence of *E. coli* F4 in ileum and colon digesta, we performed Real Time (RT) PCR by incorporating F4 fimbriae specific primers (Stahl et al., 2011). However,

we only found 2 samples showing very low amounts of the ETEC (data not shown). These results suggested that on Day 11 when piglets were sacrificed, they have shed all *E. coli* F4.

Responses of challenged piglets to probiotic feeding are species and strain dependent. Addition of *L. sorbrius* 7 days before *E. coli* F4 infection resulted in more piglets having diarrhoea symptoms in the probiotic group than in the control group. Counts of cultivable ETEC in faeces sample were not affected by diet, but RT-PCR analysis revealed a reduction of *E.coli* F4 in ileum digesta of probiotic piglets (Konstantinov et al., 2008). Similarly, feeding challenged piglets with probiotic *E. coli* strains UM-2/UM7, (Krause et al., 2010) observed reduction of ETEC counts after culturing faecal samples and gut digesta on antibiotic-supplemented plates. However, the author did not find changes in the Enterobacteriales group after T-RFLP analysis on the ileum digesta. On the other hand, inclusion of *L. rhamnosus* to post weaned piglets challenged with *E. coli* F4, resulted in enhanced diarrhoea symptoms, and higher *E. coli* F4 counts in faeces of probiotic group than in the control group (Trevisi et al., 2011). Difference levels of infection and varied piglets' sensitivities toward *E. coli* F4 among studies, including ours, may contribute to inconsistent observations regarding diarrhoea symptoms.

WP contains abundant lactose. This disaccharide is readily digestible in the ileum. The hydrolysis process from lactose to lactic acid is catalysed by brush border lactase from the piglets (Manners and Stevens, 1972)and lactase from lactose fermenting bacteria, including lactobacilli and *E. coli* (Knudsen et al., 2012). At weaning, activities of brush border enzymes are often reduced. This physiological change may be due to compromised villus-crypt structure (Kelly et al., 1991; McCracken et al., 1999). However, in this study, there was no apparent compromised of villi length in the ileum or crypt depth in the colon, except for 2 piglets in the F4 group, that showed villi atrophy at Day 11. This observation may explain the undisturbed hydrolysis of lactose to lactic acid as reflected in our result where lactic acid was dominant in the ileum of piglets from all treatment groups. Likewise, (Pieper et al., 2008) reported that 11 days post weaning, lactic acid is dominant in the small intestines of healthy piglets.

Lactic acid is an intermediate product in the mammalian gut usually found in low concentration, in faecal samples of healthy subjects. This low amount is due to further microbial conversions to butyrate, propionate or acetate, which mostly take place in colon (Belenguer et al., 2011). Indeed, the amount of lactic acid was lower in the caecum and

colon than in the ileum. Interestingly, concentrations of lactic acid were found twice or higher in the caecum of piglets fed with fermented WP relative to piglets received non-fermented WP, irrespective of the lactic acid bacteria inoculum (Table 5). An argument that additional lactic acid bacteria contributed to an increase of lactic acid was not supported by our plate counts.

In the colon, we observed that piglets fed with WP fermented with *W. viridescens* contained more acetic acid and butyric acid than piglets receiving non-fermented WP. Likewise, healthy post weaned piglets fed with multispecies probiotic showed an increased amount of acetic acid, propionic acid, and butyric acid (Mori et al., 2011). Acetic acid, propionic acid, and butyric acid are major short chain fatty acids (SCFA) and end products of colonic fermentation (Wong et al., 2006; Guilloteau et al., 2010; Knudsen et al., 2012). An increased SCFA production is linked to reduced risk of gastrointestinal disorder (Wong et al., 2006). Specifically, butyrate is preferred by the colonic epithelium where it is actively metabolised to gain energy (Guilloteau et al., 2010)..

Butyrate in colon is produced by bacterial fermentation. The butyrate-producing bacteria cultured thus far belong to strictly anaerobic firmicutes which include several clusters within the order of Clostridiales(Guilloteau et al., 2010). Accordingly, we observed an increased abundance of the phylum Firmicutes in piglets received *W. viridescens* -fermented WP. Furthermore, the there was a numerical increase of abundance of order Clostridales in this group compared to the other treatment groups.

Our study exhibited that by establishing an infection model, we were able to evaluate how piglets suffering from PWD responded to fermented WP. We observed that adding WP fermented with *W. viridescens* reduced diarrhoea incidence, improved feed intake, and the production of short chain fatty acids. These results may offer an economical yet effective preparation of probiotic feeding to post weaned piglets.

Acknowledgements

The authors are grateful for technical supports provided by the lab and animal technicians in the Foulum Experimental Station, Aarhus University and for Thomas P.B. Phil, a technician at National Veterinary Institute, DTU.

Table 1. Composition of experimental diets

Item	No WP	WP groups
Variolac 830®	0	0
Barley	20.00	20.00
Wheat	48.20	41.20
Dehuled toasted soybean meal	16.69	17.61
Animal fat	3.00	3.00
Soy protein concentrate	3.00	3.00
Potato protein	5.00	5.00
L-Lysine HCL	0.406	0.399
DL-Methionine	0.115	0.122
L-Threonine	0.096	0.098
L-Tryptophan	0.034	0.033
Monocalcium phosphate	1.322	1.242
Calcium carbonate, 38% Ca	1.198	1.185
Sodium chloride	0.505	0.419
Natruphos 5000 (100g/t)	0.013	0.013
Vitamin and mineral premix	0.40	0.40

Table 2. Chemical composition of experimental diets

Item	No WP	WP groups
Dry matter	89.1	88.8
Protein (N*6.25) % DM	21.3	22.8
Fat; % DM	5.0	5.0
Ash, % DM	4.8	5.1
Feed Unit (per kg)	116.3	113.6
Calcium (g/kg)	7.3	7.2
Fosfor (g/kg)	5.6	5.7
Valine (g/kg)	11.38	11.37
Cysteine + Cystine (g/kg)	3.71	3.90
Methionine (g/kg)	4.52	5.08
Threonine (g/kg)	9.5	10.1
Lysine (g/kg)	15.6	16.2

Table 3. The amount (g) of feed provided per meal time for each pen

Day	No WP Groups		WP Groups		
	Feed	Water	Feed	Water	WP*
1	250	625	250	400	225
2	250	625	250	400	225
3	300	750	300	480	270
4	300	750	300	480	270
5	350	875	350	560	315
6	350	875	350	560	315
7	400	1000	400	640	360
8	400	1000	400	640	360
9	450	1125	450	720	405
10	450	1125	450	720	405
11	500	1250	500	800	450

* non-fermented or fermented

Table 4. Growth performance of piglets fed the experimental diets

Item	Treatment						SEM	*P*-value
	No F4	F4	F4+WP	F4+WP+ Pro1	F4+WP+ Pro2	F4+WP+ Pro3		
ADG, g								
1 to 4 days	991.67	521.25	948.00	751.25	907.00	1324.00	108.90	0.696
1 to 8 days	1793.33	1500.00	2187.00	1160.00	1893.00	2297.00	173.68	0.853
1 to 11 days	2986.67	2811.25	3665.00	2116.67	3237.00	3378.00	219.93	0.655
ADFI, g								
1 to 4 days	1004.30	881.09	884.90	393.05	848.34	1007.48	92.83	0.161
1 to 8 days	2802.41	2550.14	2727.88	1433.97	2586.64	2822.13	215.45	0.054
1 to 11 days	3745.71[a]	3448.25[ab]	3706.47[a]	2128.08[b]	3533.52[a]	3789.50[a]	258.37	0.018

Data is presented as least-square means (n=10).

[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

SEM, standard error of the mean.

Table 5. The concentration of organic acids (mmol/kg) and pH in the digesta from the gastrointestinal tract of piglets fed experimental diets

	Treatment						SEM*	*P*-value		
	No F4	F4	F4+WP	F4+WP+Pro1	F4+WP+Pro2	F4+WP+Pro3		Treatment	Segment	Treatment x segment
Lactic acid								0.350	< 0.001	0.517
Stomach	26.60	21.12	31.85	41.75	49.89	50.92	5.06			
Ileum	20.82	35.83	36.93	35.39	26.10	27.59	2.68			
Caecum	0.51	2.57	3.19	7.97	14.42	12.45	2.32			
Colon	2.60	0.23	0.17	0.00	0.00	0.23	0.41			
Formic acid								0.530	< 0.001	0.278
Stomach	0.00	0.00	0.31	0.00	0.00	0.34	0.07			
Ileum	2.61	11.21	8.52	8.59	2.98	4.83	1.43			
Caecum	1.28	0.81	0.34	0.31	0.21	1.13	0.18			
Colon	2.01	0.49	0.19	0.00	0.87	0.43	0.29			
Acetic acid								0.026	< 0.001	0.343
Stomach	10.19	7.80	6.70	6.42	5.81	9.33	0.71			
Ileum	4.97	8.38	11.38	10.05	6.18	8.70	0.97			
Caecum	59.32	65.47	76.49	81.95	66.88	75.31	3.43			
Colon	45.35^{c}	49.36^{bc}	55.22^{abc}	73.16^{a}	63.01^{abc}	71.80^{a}	4.73			
Propionic acid								0.019	< 0.001	0.116
Stomach	1.58	1.51	0.08	0.13	0.00	2.26	0.71			
Ileum	0.07	0.00	0.00	0.00	0.00	0.00	0.01			
Caecum	23.63^{b}	27.94^{ab}	34.94^{a}	38.41^{a}	33.02^{ab}	31.98^{ab}	2.13			
Colon	17.43^{c}	19.54^{bc}	24.36^{bc}	29.70^{ab}	25.73^{abc}	33.39^{a}	2.45			
Butyric acid								0.032	< 0.001	0.212
Stomach	1.06	1.58	0.24	0.12	0.04	0.39	0.25			
Ileum	0.13	0.20	0.38	0.37	0.35	0.36	0.04			
Caecum	7.46	9.63	9.37	12.99	7.69	11.64	0.89			
Colon	6.34^{b}	7.96^{b}	8.44^{b}	14.05^{a}	10.26^{ab}	13.20^{a}	1.25			

Item	Treatment						SEM*	*P*-value		
	No F4	F4	F4+WP	F4+WP + Pro1	F4+WP +Pro2	F4+WP +Pro3		Treatment	Segment	Treatment x segment
Valeric acid								0.034	< 0.001	0.484
Stomach	0.31	0.52	0.00	0.00	0.00	0.10	0.09			
Ileum	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Caecum	0.91^{b}	1.54^{ab}	0.83^{b}	2.71^{a}	0.59^{b}	1.16^{b}	0.31			
Colon	1.36^{b}	1.75^{ab}	1.41^{b}	3.07^{a}	1.58^{b}	1.63^{ab}	0.26			
Isobutyric acid								0.099	< 0.001	0.003
Stomach	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Ileum	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Caecum	0.21	0.26	0.34	0.13	0.09	0.29	0.04			
Colon	1.12^{bc}	1.21^{b}	1.15^{b}	1.62^{a}	1.04^{bc}	0.85^{c}	0.11			
Isovaleric acid								0.129	< 0.001	0.009
Stomach	0.31	0.52	0.00	0.00	0.00	0.10	0.09			
Ileum	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Caecum	0.13	0.08	0.14	0.00	0.03	0.12	0.02			
Colon	0.73^{b}	0.73^{b}	0.76^{b}	1.19^{a}	0.75^{b}	0.48^{b}	0.09			
Acetic + propionic + butyric acid								0.011	< 0.001	0.164
Stomach	12.83	10.89	7.02	6.67	5.85	11.99	1.24			
Ileum	5.17	8.57	11.75	10.42	6.53	9.06	0.99			
Caecum	90.41^{b}	103.03^{ab}	120.80^{ab}	133.35^{a}	107.59^{ab}	118.93^{ab}	6.18			
Colon	69.13^{c}	76.86^{c}	88.07^{bc}	116.09^{ab}	99.01^{abc}	118.38^{a}	7.08			
pH								0.026	< 0.001	0.136
Stomach	3.82	3.41	3.94	3.40	3.89	3.95	0.11			
Ileum	7.15^{a}	6.44^{b}	6.66^{ab}	6.65^{ab}	6.94^{ab}	6.76^{ab}	0.10			
Caecum	6.52^{a}	6.39^{ab}	6.13^{ab}	5.93^{ab}	5.88^{ab}	6.09^{ab}	0.10			
Colon	6.67	6.40	6.52	6.67	6.43	6.53	0.05			

* SEM = pooled standard error of mean

^{a,b,c} Means within rows without a common letter differ (P<0.05)

Table 6. Counts of selected microbial populations (log cfu/g of digesta) in digesta from the gastrointestinal tract of piglets fed experimental diets

Segment	Treatment						SEM	*P*-value	
	No F4	F4	F4+WP	F4+WP+ Pro1	F4+WP+ Pro2	F4+WP+ Pro3		Diet	Diet x segment
Lactic acid bacteria								0.059	0.349
Stomach	7.98	8.65	8.50	8.51	8.65	8.47	0.10		
Ileum	8.12	8.56	8.75	9.03	8.55	8.51	0.12		
Caecum	8.88	8.96	8.80	9.03	8.76	8.93	0.04		
Colon	9.23	9.23	9.12	9.19	9.09	9.20	0.02		
Yeasts								0.687	0.884
Stomach	5.15	4.68	5.11	4.70	5.11	4.96	0.09		
Ileum	4.46	4.90	4.49	5.15	5.18	4.84	0.11		
Caecum	4.31	5.19	4.64	5.20	4.61	4.43	0.15		
Colon	4.82	4.63	4.31	5.06	4.36	3.99	0.16		
Enterobacteriaceae								0.093	0.803
Stomach	5.68	6.04	5.60	5.12	5.61	5.34	0.13		
Ileum	7.41	7.72	7.11	6.71	7.05	6.87	0.15		
Caecum	7.66	7.35	7.40	7.64	6.77	7.41	0.13		
Colon	7.95	7.46	7.43	7.12	6.92	6.99	0.16		
Total anaerobic bacteria								0.420	0.131
Stomach	7.20	8.30	7.85	8.14	7.51	7.89	0.17		
Ileum	8.03	8.52	8.60	8.56	8.39	8.36	0.09		
Caecum	8.96	9.27	9.27	8.87	8.99	8.99	0.07		
Colon	9.40	9.37	9.24	9.19	9.62	9.57	0.07		

Data is presented as least-square means (n=10).

SEM, SE of the mean.

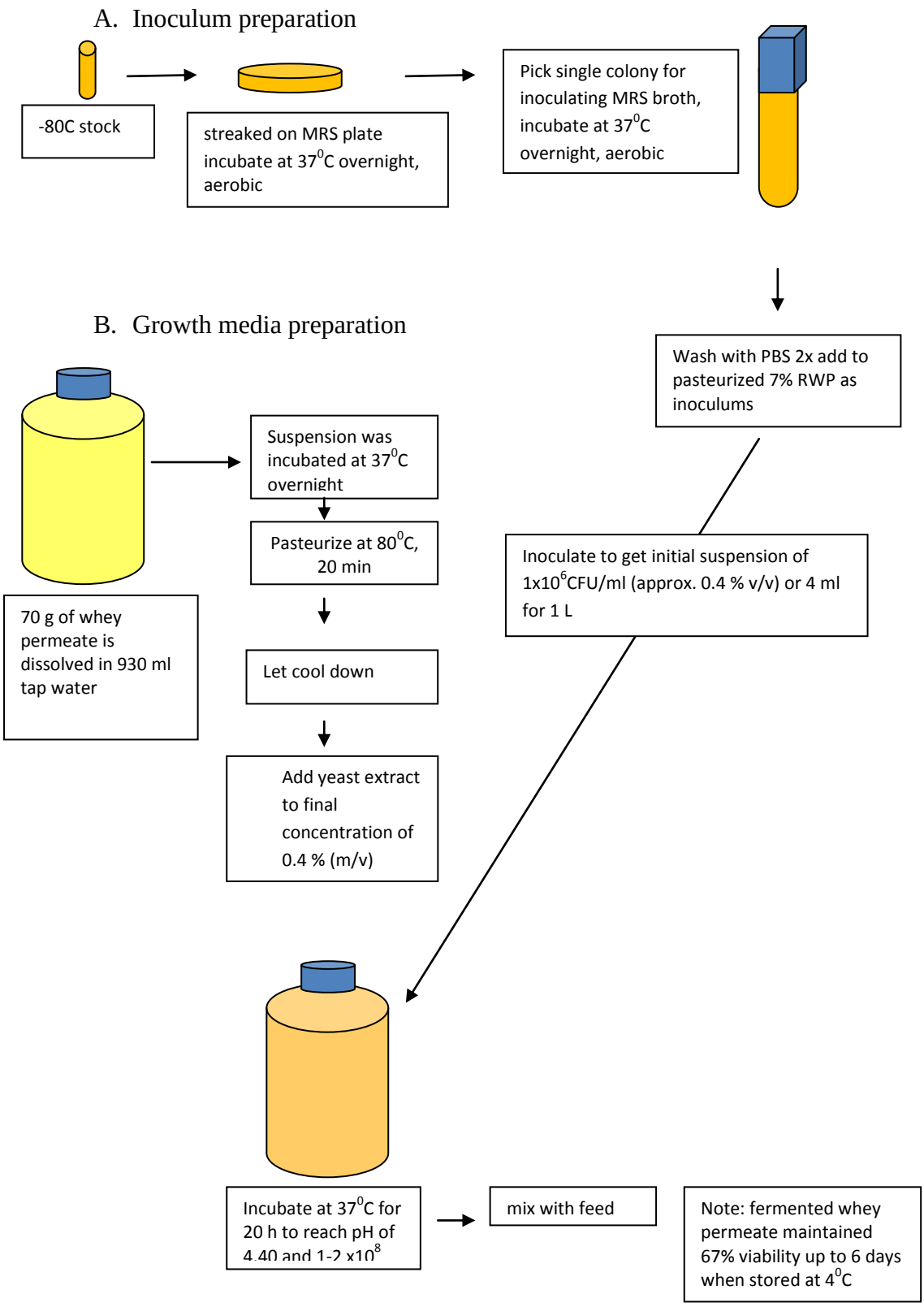
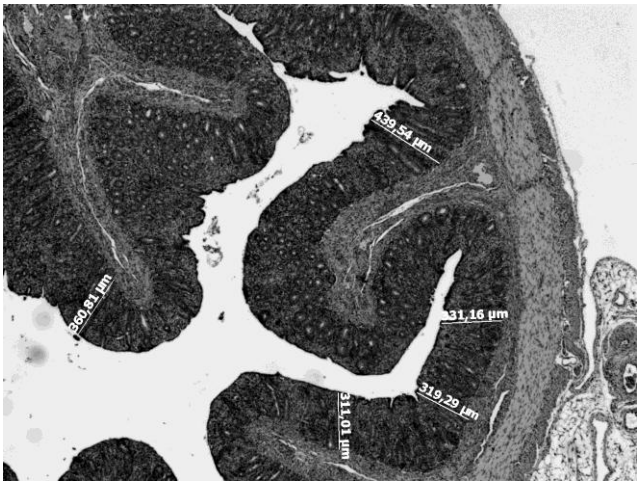


Figure 1. Schematic steps of fermented whey preparation using *L. plantarum* or *W. viridescens* as inoculums

a.



b.

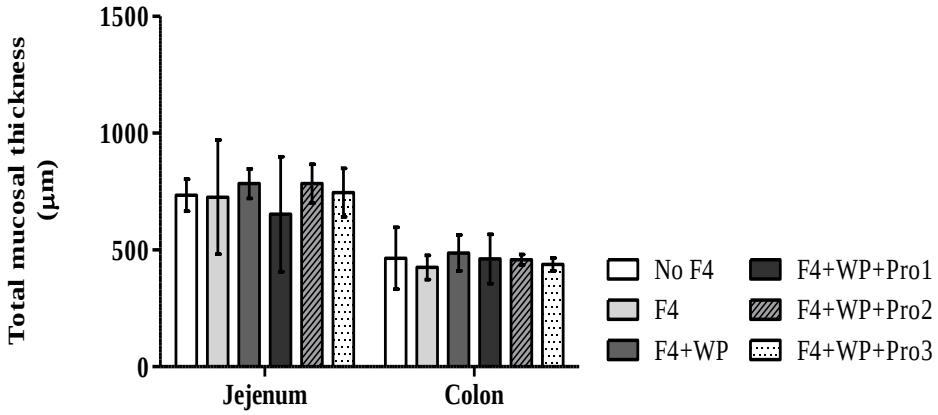
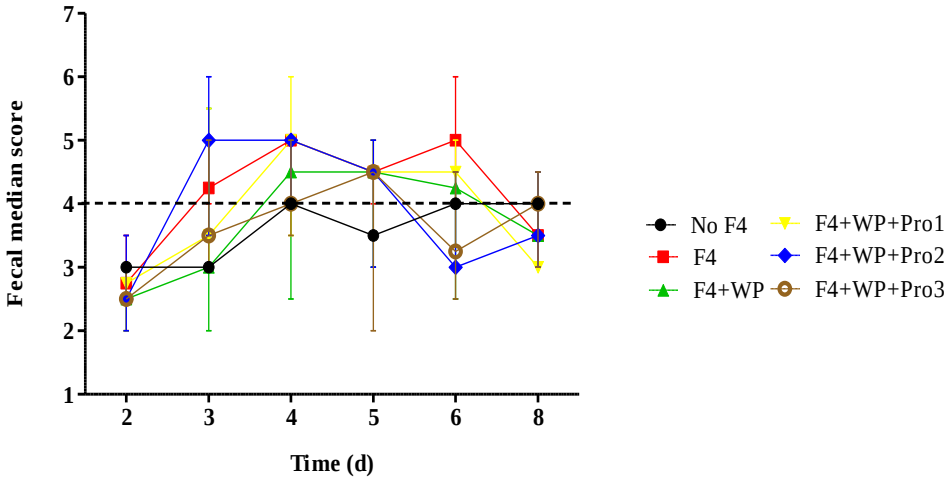
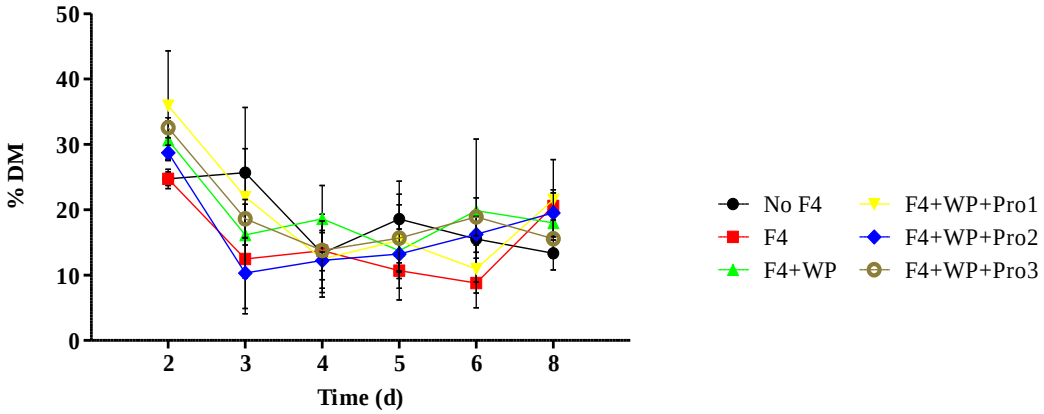


Figure 2. Histomorphometry of gastrointestinal parts of piglets fed with experimental diets. (a). Villi atrophy in piglet number 33 observed under the microscope (2.5 x Obj magnification power) (b). Total mucosal thickness of jejenum and colon

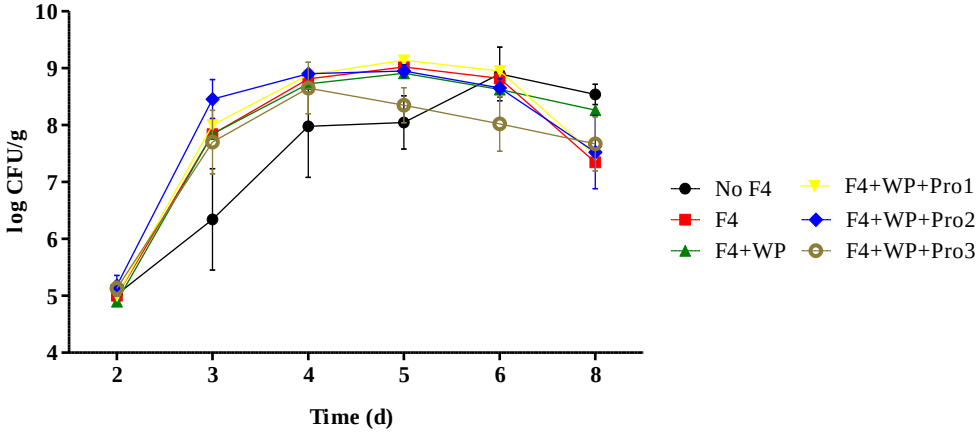
a.


Fecal median score
Time (d)
No F4
F4
F4+WP
F4+WP+Pro1
F4+WP+Pro2
F4+WP+Pro3

b.


% DM
Time (d)
No F4
F4
F4+WP
F4+WP+Pro1
F4+WP+Pro2
F4+WP+Pro3

c.


log CFU/g
Time (d)
No F4
F4
F4+WP
F4+WP+Pro1
F4+WP+Pro2
F4+WP+Pro3

d.

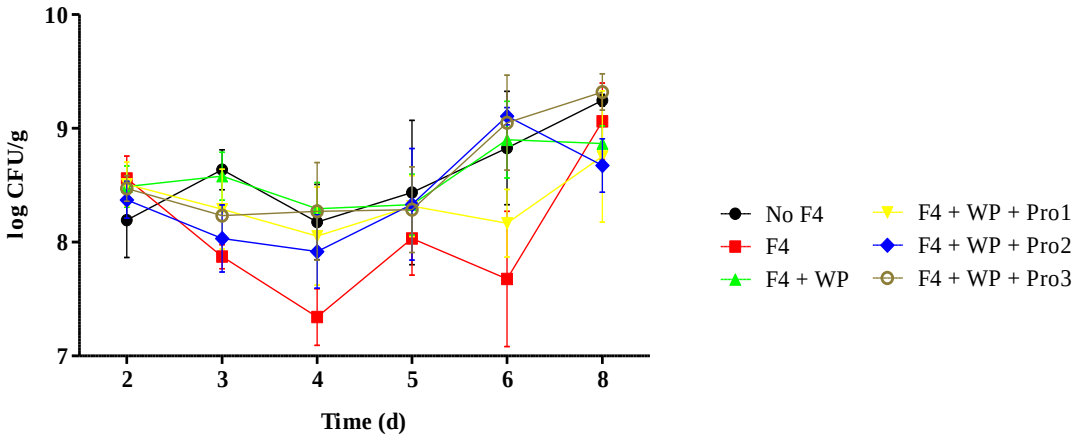
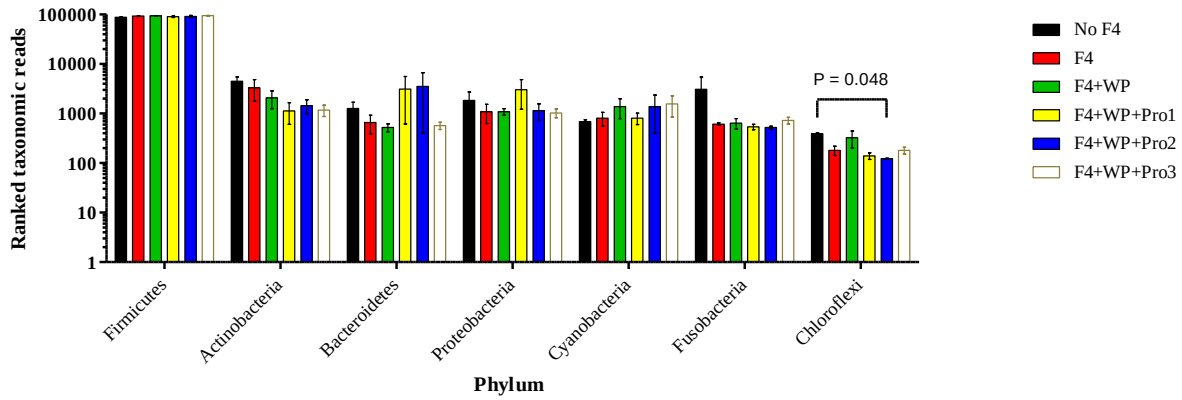


Figure 3. Analyses of fecal samples from piglets fed with treatment diets on different days of the experiment (a). Fecal score, 4 or above indicated diarrhoea symptom (b). % Dry matter (c). Counts of hemolytic coliform and (d). Counts of lactic acid bacteria. Each point represents mean value for each treatment group within the same day.

a.



b.

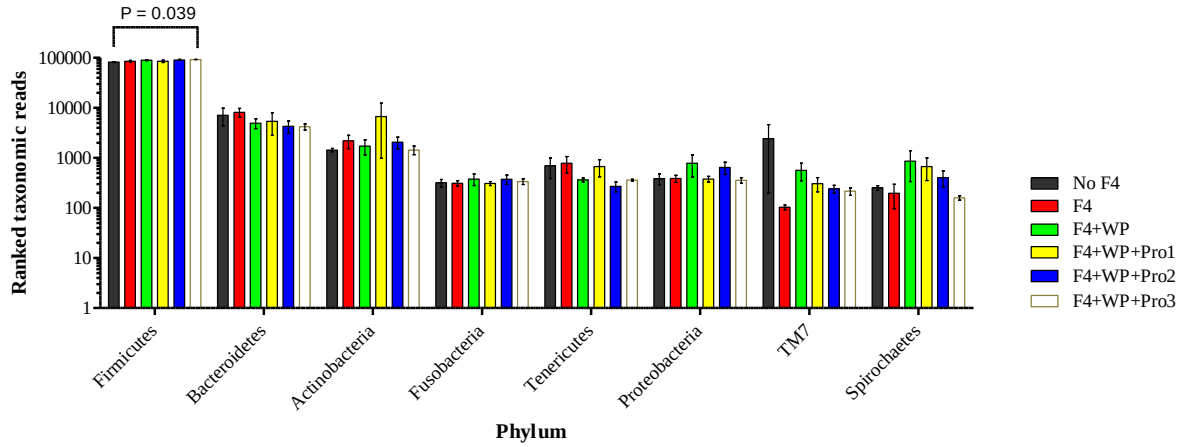


Figure 4. Microbiota community structure at phyla level represented by the most abundant phylain (a) ileum and (b) colon digesta of piglets fed with experimental diets. Value was expressed as ranked taxonomic reads normalized to 100,000.

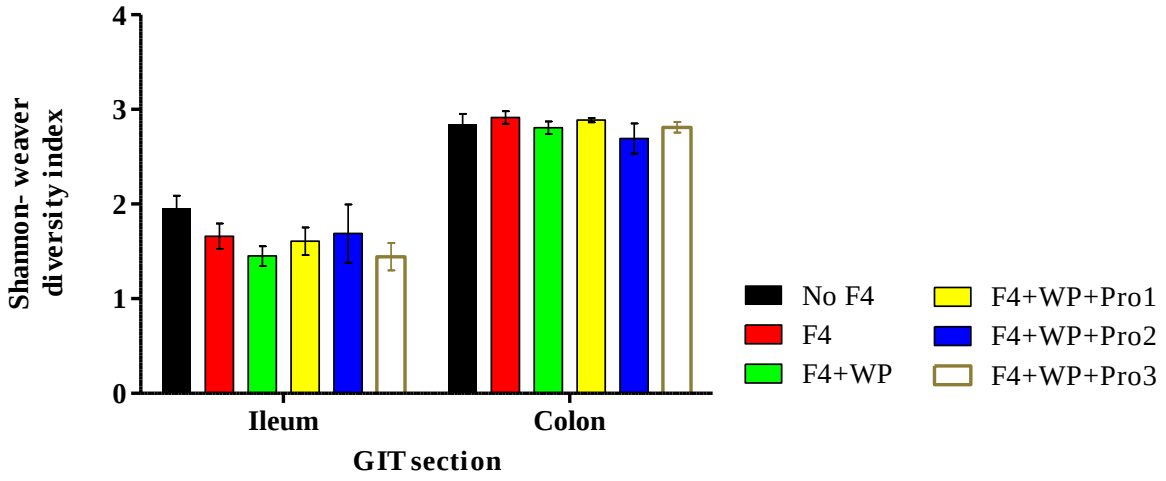


Figure 5. Diversity of bacterial community in digesta samples of ileum and colon from piglets fed with experimental diet expressed as Shannon-weaver indices Significance among colon and ileum?

Paper 4. Early draft

Extracts of *Fructus mume* inhibit *E. coli* F4 and modulate the innate immune response in IPEC-J2 cells

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Running title: *Fructus mume* effects on *E.coli* and innate immune response

Keyword: Fructus mume, *E. coli*, IL-18, TLR-4, IPEC-J2 cell line

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Abstract

Extracts *of Fructus mume* (FM) and *Ziziphi spinosa* (ZS) have been used among Chinese medical remedies for many generations. *Fructus mume* has been reported to inhibit pathogens and ZS may induce appetite, which together as a mixture, might help in mitigating stress symptoms and reduce post weaning diarrhoea (PWD) among piglets. This study aims to determine the minimal inhibitory concentration (MIC) of *FM* and *ZS* ethanolic extracts against post weaning diarrhoea relevant *E. coli* F4 and to investigate the response of innate immune genes to these extracts by a porcine intestinal epithelial cell line (IPEC J2) *in vitro*. Extracts were prepared individually or as a 9:1 (FM:ZS) mixtures. Microdilution method was used to determine MIC values. Expression of mRNA coding for innate immune genes were determined by quantitative high throughput real-time reverse transcription polymerase chain reaction (RT-qPCR). *Fructus mume* inhibits *E. coli* F4 growth? at 1 mg/ml and as a mixed extract at 2.5 mg/ml. *Ziziphi spinosa* did not inhibit *E. coli* F4. Expression of TNF-α and IL-18 genes in IPEC J2 cells was down regulated by both the mixed extract and the FM extract, whereas the TLR-4 gene was up regulated in the IPEC-J2 cell line**.** Further we observed subtle but significant down-regulation of IL6, IL8 and NOD1 in response to mixed extracts or FM extract. The study demonstrated that *Fructus mume* extract may play a role in inhibiting *E. coli* F4 infection and regulating pro-inflammatory cytokine responses, while ZS may have less measurable contributions when the mixed extract of this CHM is applied in post weaning diarrhoea management.

1. Introduction

Two of Chinese herbs that have been frequently used as main ingredients in the development of medicinal extracts are *Fructus mume* and *Ziziphus jujube* Mill. var *spinosa.* Traditionally in China, Japan, and South Korea, the extracts of *Fructus mume* fruit is applied as folk medicine, assuages fever, cough, diarrhea and other intestinal disorders. On the other hand, the extract from *Ziziphus spinosa* has been widely used to treat insomnia (Choi et al., 2007; (Chuda et al., 1999; Jiang et al., 2007). Different from Western medicine, most Chinese herb extracts are compounded and formulated specifically to accommodate the patient's conditions. Accordingly, various *in vitro* and clinical studies have reported the efficacy of Chinese herb extracts in the form of mixtures (Lee and Stein, 2011; Ding Y. et al. 2011). Scientific information of the effects of the herb extract when applied separately or in combination is limited.

Fructus mume possesses antimicrobial properties when evaluated as independent extract or when it is being part of mixed extract (Nakajima et al., 2006; Lee and Stein, 2011; Seneviratne et al., 2011; Xia et al., 2011). However, when isolated, the phenolic compounds from *Fructus mume* exhibited less antimicrobial activities compared to the original crude extract (Xia et al., 2011). Meanwhile, the extracts of *Ziziphus spinosa* seed modulates IL-1β, IL-4, IL-6, IL-10, TNF-α and IFN-γ as measured from mouse serum. These cytokines are linked to hypnosis effects in mice (Xie et al., 2011). Furthermore, a, feeding the mixture of *Fructus mume* and *Ziziphus spinosa* extract at 200 mg/kg body weight protected chickens from *E. coli* infections, decreased viral infections, and reduced the mortality rate (Li et al. 2010a, 2010b)

Post weaning diarrhoea (PWD) is a global problem which continuously causes economic burden in the swine industry. The disease has been associated with bacterial infections, which result in scouring, poor growth performance, upregulation of inflammatory cytokines in piglets, and in extreme cases even mortality. *E. coli* F4 is often related to PWD worldwide (Pie et al., 2004; Fairbrother et al., 2005; Bhandari et al., 2008). Systematic efforts to solve this problem include the administration of Chinese Herbal Medicine (CHM) (Ding et al., 2011).

Fermented *Fructus mume* induced expression of TNF-α and IFN-γ in spleen of experimental mice infected with *B. bronchiseptica*, a pathogen which is often found in the respiratory tract of a variety of mammalian species, especially swine (Jung et al., 2010). However, whether the extract of *Fructus mume* or *Ziziphi spinosa* semen (seed) affect the immune system in the gastrointestinal of swine is still unclear. Intestinal Porcine Epithelial Cell-J2 (IPEC-J2) provides a reliable *in vitro* system to study immune responses by porcine epithelial cells after treatment with chemical substances or when exposed to bacterial infections (Schierack et al., 2006). This experiment aims to determine the Minimal Inhibitory Concentrations (MICs) of *Fructus mume* fruit and *Ziziphus spinosa* seed, as separate or mixed ethanolic extracts, that inhibit *E. coli* F4 and to assess responses of immune genes by the epithelial cells after being treated/exposed to extracts *in vitro*.

2. Materials and Methods

2.1 E. coli F4 and culture medium

Escherichia coli 9910045-1:0149ST2,LT,F4ac (hereafter will be written as *E. coli* F4), was first isolated at the Danish Veterniary Insitute (Frydendahl et al., 2001). For each experiment, a fresh culture was prepared from -80 ºC frozen collection streaked on McConkey (Difco) plate. The plate was incubated at 37 ºC for 16 h. A single colony was picked to inoculate a tube containing 10 ml Brain Heart Infusion (BHI) media (Merck). Inoculated BHI was incubated at 37 ºC for 16 h. The optical density of overnight suspension (OD_{600nm}) was determined, and 10-fold serial dilutions in 0.85% saline were performed to enumerate *E. coli* F4.

2.2 Preparation of Chinese herbal extracts

Ziziphi spinosa (ZS) seed was treated as previously described. The seeds were grinded and were soaked in petroleum ether to remove lipids. The mixture was stirred occasionally. Skimmed ZS was dried at room temperature and then mixed with FM at the ratio of 1:9. The mixture was soaked in 75% ethanol and was heated in an electronic temperature regulation heating mantle until boiling. The extraction proceeded for 2 h. The resulting extract was concentrated under reduced pressure in a rotary evaporator (Shanghai Yarong). Syrup was finally obtained and kept at 4^0C until use. Preparation of individual exract followed the same extraction procedure without mixing the two sources.

2.3 MIC determination

Stock and working solution of CHM extracts

A 300 mg/ml stock solutions for ZS and FM extract were prepared by mixing 15 g of respective syrup extract and 20 ml of warm 30% ethanol, into which deionized water was added to make a 50 ml total solution. Stock solutions were kept at 4 ºC until use. Working solutions were prepared by dissolving stock solution in 20% (v/v) ethanol to reach desired concentration. Ethanol final concentration in each well was maintained to be < 3 % (v/v) to avoid ethanol toxicity towards bacterial cells. The concentrations of *Ziziphi spinosa* (ZS) semen extract tested ranged were 0 to 40 mg/ml whilst the range for *Fructus mume* (FM) extract and mixture (MIX) of both herbs extracts was from 0 to 20 mg/ml.

The resistance of *E. coli* F4 to CHM extracts was determined by standard broth microdilution (Wiegand et al., 2008)(Palaniappan and Holley, 2010)with minor modifications. Mueller Hinton broth (MHB) (Oxoid, Fisher Scientific) was used to refresh *E. coli* a day prior to the MIC experiment. Experiments were performed in 96 wells microplates (Nunclon, Nunc A/S, Roskilde, Denmark) with a total volume of 100 µl in each well. Treatment wells were filled with 90 µl of *E. coli* F4 suspension containing 1 x 10^5 CFU and 10 µl of plant extracts. The study included Ampicillin, Neomycin, and Streptomycin, and ethanol 3% as controls. Filled microplates were sealed with sterile plate cover (Nunc A/S, Roskilde, Denmark) and placed on a shaker with 150 rpm speed. The shaker was placed in a 37^0C incubator for 16 h. After reading the turbidity (ThermoFisher), 50 µl of aliquot was taken for plating on Luria Bertani (LB) plates. Plates were incubated at 37 ºC for 16 h and enumerated. Each treatment level was performed in 3 separate wells. The MIC of respective plant extract was determined as the lowest concentration that significantly suppressed the growth of *E. coli* F4.

2.4 Growth and maintenance of IPEC-J2 cell line

The IPEC-J2 cell lines (Intestinal Porcine Epithelial Cell Jejunum) are originally collected as a non-transformed intestinal cell line from jejunum epithelium isolated from a neonatal, non suckled piglet and was maintained as previously described (Schierack et al., 2006). Briefly, a cell line from a -80 ºC collection was grown in 15 mL Dulbecco's modified eagle medium (DMEM:F12 = 1:1, Merck, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Merck, Germany), 2 mM L-Glutamine (Sigma), 1 mM pyruvate, 100 U/ml penicillin

and 100 µg/ml streptomycin (Sigma). The cell line was cultivated at 37 ºC under 5% CO_2 atmospheric level and 95% air. Cell growth media were replaced every second day and monolayer cell lines were routinely subcultured every 7 days. Prior to studies, cell lines were routinely tested to be free from mycoplasma.

2.5 Studies on IPEC-J2 cell line.

An amount of 900 µl IPEC-J2 cells per well were added into 12-well plates (Nunc, Roskilde, Denmark) to reach approximately 1.5 x 10^5 cells per well. The cells were allowed to adhere and reach 90% confluence at 37 ºC under 5% CO_2 prior to extract additions. The following ranges of final extract concentrations were tested: 0.25 to 5.0 mg/mL ZS (ZS 0.25; ZS 2.5; and ZS 5); 1.0 to 5.0 FM (FM 1; FM 2.5; and FM 5) and MIX (MIX 1; MIX 2.5; and MIX 5). Positive control wells were prepared to contain 1 µg/ml of Lipopolysaccharide (LPS) from *Salmonella enterica* serotype Typhimurium (Sigma Life Science, Brøndby, Denmark). Cells were allowed to be in contact with extracts for 3 h at 37 ºC under 5% CO_2 atmospheric level and 95% air.

Carefully, growth media were collected from each well. Detached cells were collected as pellet after centrifugation (400xg/10 min/4 ºC). The attached cells in the wells were washed twice with 1xPBS and released by 0.04% trypsin. The cell suspension was added into the pellet tube. The tube, which contained both detached and attached cells, was centrifuged (400 x g/10 min/4 ºC). Cells were resuspended in RLT-buffer (Qiagen, Albertslund, Denmark) containing 1% β-mercaptoethanol and were stored at -80 ºC for RNA extraction.

RNA extraction, cDNA synthesis and pre amplification

Total RNA from IPEC-J2 cells was extracted using Rneasy Mini kit (Qiagen) and treated using Rnase-free Dnase kit set (Qiagen) following manufacture's recommendation. Quantity and quality of extracted total RNA was measured as described previously (Skovgaard et al.,2010)

Extracted RNA was converted into cDNA by reverse transcription of total RNA using the QUantiTECT Reverse Transcription kit (Qiagen), containing a mix of random primers and oligo-dT, according to the manufacturer's instructions. The cDNA product was diluted 1:7 in low EDTA TE-buffer (VWR – Bie & Bernsten) prior to pre amplification. Pre amplification

was performed as previously described (Soerensen et al., 2012) Pre amplified cDNA was diluted at least 1:4 in low EDTA TE-buffer (VWR – Bie & Bernsten) before RT-qPCR.

Quantitative real time PCR (qPCR)

Quantitative PCR was performed in 48.48 Dynamic Array Intergrated Fluidic Circuits (Fluidigm, CA, USA.), combining 48 samples with 48 primer sets for 2304 simultaneous qPCR reactions as previously described (Soerensen et al., 2012). Primer design and validation was performed as described in Skovgaard et al., (2009), (primers can be seen in Table 1). After loading and mixing of each sample-primer reaction in individual chambers, the Dynamic Array was placed in the BioMark real-time PCR instrument (Fluidigm). The following cycle parameter was used: 2 min at 50 ºC, 10 min at 95 ºC, followed by 35 cycles of 15 s at 95 ºC and annealing/elongation for 1 min at 60 ºC. Melting curves were generated after each run to confirm a single PCR product (from 60 ºC to 95 ºC, increasing rate of 1 ºC/3 s). Non template controls (NTC) were included to monitor any potential problems with non-specific amplification or sample contaminations. Non-reverse transcriptase controls were included to assess potential DNA contamination. Expression data (Cq) were obtained using the Fluidigm Real Time PCR Analysis software 3.0.2 (Fluidigm) and exported to GenEx (MultiD). Using non-amplified cDNA, a second qPCR was performed on the RotorGene 3000 Detection System (Corbett Research, Sydney, Australia) to validate selected expression differences found in the Fluidigm platform. The following conditions were used on the RotorGene 3000: 2 min at 50 ºC, 10 min at 95 ºC, followed by 40 cycles with 15 s at 95 ºC and 1 min at 59–62 ºC.

IPEC J2 gene expression

Data pre-processing, normalisation, relative quantification and statistics were performed using GenEx5 (MultiD). Data was pre-processed as follows: 1. Data was corrected for PCR efficiency for each primer assay individually; 2. Normalisation was done to the mean of six reference genes (GAPDH, HPRT1, RPL13A, PPIA, TBP and YWHAE (Table 1) found to be the most stably expressed reference genes out of seven tested in the present study using both GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). Expression for all samples was calculated relative to the average expression of the control group for each primer assay. For graphical presentation, the normalized mean for the growth media group

was set to one, and the normalised means from the other four groups were displayed as fold change compared to the normalised mean to the growth media group. Statistical analysis was performed using ANOVA. Differences between treatment groups were further determined by t-test on non-transformed and $\log_2$-transformed data, and only significant differences occurring in both analyses were considered significant. Significant difference was obtained from means giving P-value < 0.05.

3. Results

3.1 *Antimicrobial activity against E. coli* F4

In this part of the experiment, we aimed to determine the concentration at which the Chinese herbal medicine (CHM) extract, individually or as a mixture significantly inhibit *E. coli* F4 *in vitro*. The results showed that CHM extracts inhibited *E. coli* F4 at different concentrations (Figure 1). *Fructus mume* (FM) extracts significantly inhibited the *E. coli* F4 at a concentration of 1 mg/ml. *Ziziphi spinosa* (ZS) extract did not inhibit the indicator pathogen, even up to 40 mg/ml. However, the extract of mixed Chinese herb (MIX) showed inhibition at 2.5 mg/ml, which is a higher MIC value compared to FM activity. *E.coli* F4 was completely inhibited at concentration of FM or MIX at 20 mg/ml.

3.2 In-vitro innate immune gene responses

In this experiment, we wanted to evaluate the expression of Toll like receptor 4 (TLR-4) and other pattern recognition receptors, pro- and anti- inflammatory cytokines as well as other antimicrobial molecules by IPEC-J2 cells. A total of 18 genes of interest were evaluated (IL-1A, IL-1RN, IL-6, IL-8, IL-18, TNF, TLR-2, TLR-3, TLR-4, CD14, DEFB1, defensin, MD2, MUC1, NOD1, PAFA, SAA, and TGFb). Significant effects were observed on TLR-4 ($P = 0.005$) and IL-18 ($P = 0.0115$) when compared to expression in the control group after statistical t-test. These results were validated on non-amplified cDNA using a second qPCR platform (see Materials and Methods). Subtle changes in expression of mRNA coding for several other transcripts including IL-6, IL-8 (data not shown), and NOD1 (Figure 3) were further found.

At 5 mg/ml, *FM* significantly increased expression of TLR-4 ($P = 0.005$) (Figure 2). Similarly, when mixed with *ZS*, CHM increased TLR-4 expression at a final concentration of 1 mg/ml ($P = 0.011$) or 2.5 mg/ml ($P = 0.003$). These observations were consistent across two different primer pairs targeting TLR-4 (Table 1). On the other hand, expression of IL-18 decreased gradually as the concentration of mixed extract being added on IPEC-J2 cells increased. These results are based on the different highly correlated primer pairs amplifying two separate locations on the IL-18 transcript.

At 5 mg/ml, FM significantly suppressed IL-18 expression ($P = 0.05$) which was also showned by the cells after in contact with/exposed to 5 mg/ml MIX at for 2 hours ($P = 0.0247$) (Figure 2b).

Likewise, TNF-α was down regulated by MIX 1 (P = 0.003) and MIX 2.5 (P = 0.005). On the other hand, all extracts down regulated NOD1 (P = 0.016).

In addition, IL-6 and IL-8 expressions were down regulated after exposure to MIX 1 and 2.5 as well as FM 5. However, these observations on IL-6 and IL08 were not statistically significant with P = 0.13 and P = 0.058, respectively. In this study, we could not measure the immune responses from ZS treatments due to lack of enough mRNA materials.

4. Discussion

The Chinese pig production is the biggest in the world and including CHM extracts in animal feed, is a growing practice among Chinese farmers. However, as these additives are produced as compounded mixture, limited information is available on how individual extract confer benefits. The knowledge about extract performance, as a separate entity or as a mixture would accommodate the need to find the active components in the extracts for future applications. The present study demonstrated that ethanolic extracts of *Fructus* FM individually or as mixed extracts inhibit *E. coli* F4, a pathogen that is widely related to PWD in piglets (Frydendahl et al. 2003), and was further found to repress the expression of several pro-inflammatory cytokines in porcine epithelial cells *in vitro*.

The results indicated that, between the two extracts, only FM extract was antagonistic against the enterotoxigenic bacteria. In accordance with this study, FM as part of a herbal combination has previously reported inhibiting entherohemorrhagic (EHEC) *E. coli* (Lee and Stein, 2011). Furthermore, seed extract of FM has been found to be inhibitory against non pathogenic *E. coli* (Xia et al. 2011). These authors further hypothesized that the antimicrobial activities were attributed to the synergism of chlorogenic acid isomers and other components in the extract (Xia et al. 2011). Whereas most of the ZS applications are not related to pathogenic infections, but reportedly are for insomnia treatments (Jiang et al. 2007; Xie et al. 2011).

Fructus mume (FM) individually or as mixed extracts with ZS might process anti-inflammatory or immune modulating activities. The IPEC-J2 cell line has previously proved to be an excellent in vitro system for studies of infection processes in intestinal epithelial of swine (Schierack et al., 2006). FM suppressed the expression of IL-18. However, only as mixture FM also reduced TNF-α expression. Both IL-18 and TNF-α belong to the pro-inflammatory cytokines. Over expression of

TNF-α relaxes tight junction, which may increase the risk of *trans*-epithelial pathogen invasion (McKay and Baird, 1999; Pie et al., 2004). Pro-inflammatory cytokines are of great importance in initiating an efficient immune response, but must be tightly regulated.

Among post weaned piglets, the increase of IL-18 occurs later after weaning (day 8) compared to TNF-α (day 2) (Pie et al., 2004). The authors suggested that regulating expressions of pro-inflammatory cytokines at different age after weaning help in reducing the propensity of piglets to develop diarrhoea. FM individually or as a mixture might possess these features, but further studies need to be performed to elucidate the in vivo dose effect. Further the FM significantly increased expression of TLR-4. Toll like receptors, including TLR-4, are proteins on intestinal epithelial cells which are involved in protection from pathogens through the induction of IgA and antimicrobial peptides as part of the innate immunity (Abreu, 2010; Devriendt et al., 2010). The role of TLR signaling in the epithelium of the digestive tract has not yet been fully elucidated, but TLR-4 is commonly reported as been activated by Gram-negative lipopolysaccharides (Abreu, 2010).

Contacts with extracts of MIX showed repression of IL-18 and TNF-α and an increased expression of TLR-4, respectively. Combining with the results from MIC values, MIX extract at 1 mg/ml may be relevant in PWD management, by reducing the numbers of *E. coli* and at the same time priming the immune system right after the pigs are weaned. Furthermore, our results indicated that including ZS in the mixed extract did not contribute in *E. coli* F4 inhibition nor regulating immune response.

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Figures

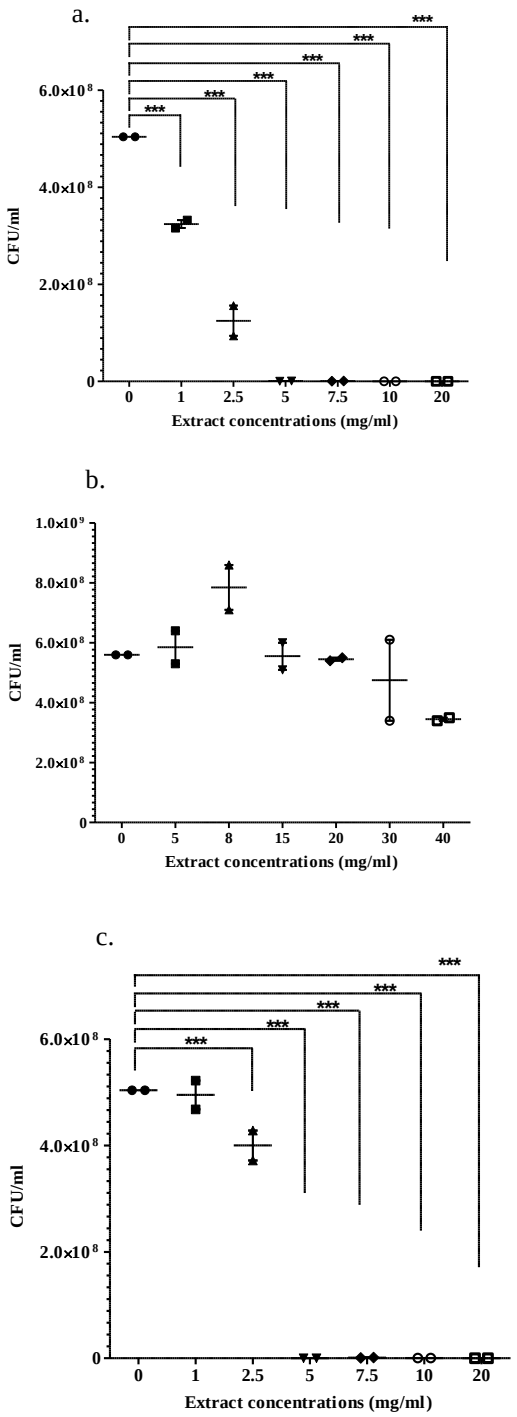

Figure 1. Changes in *E.coli* F4 count after being in contact with different concentrations of Chinese Herb Medicines (CHM) a. *Fructus mume*; b. *Ziziphii spinosa* semen; c. Mixture of the two extracts. Mean values with different alphabetical symbols were significantly different *** ($P < 0.001$)

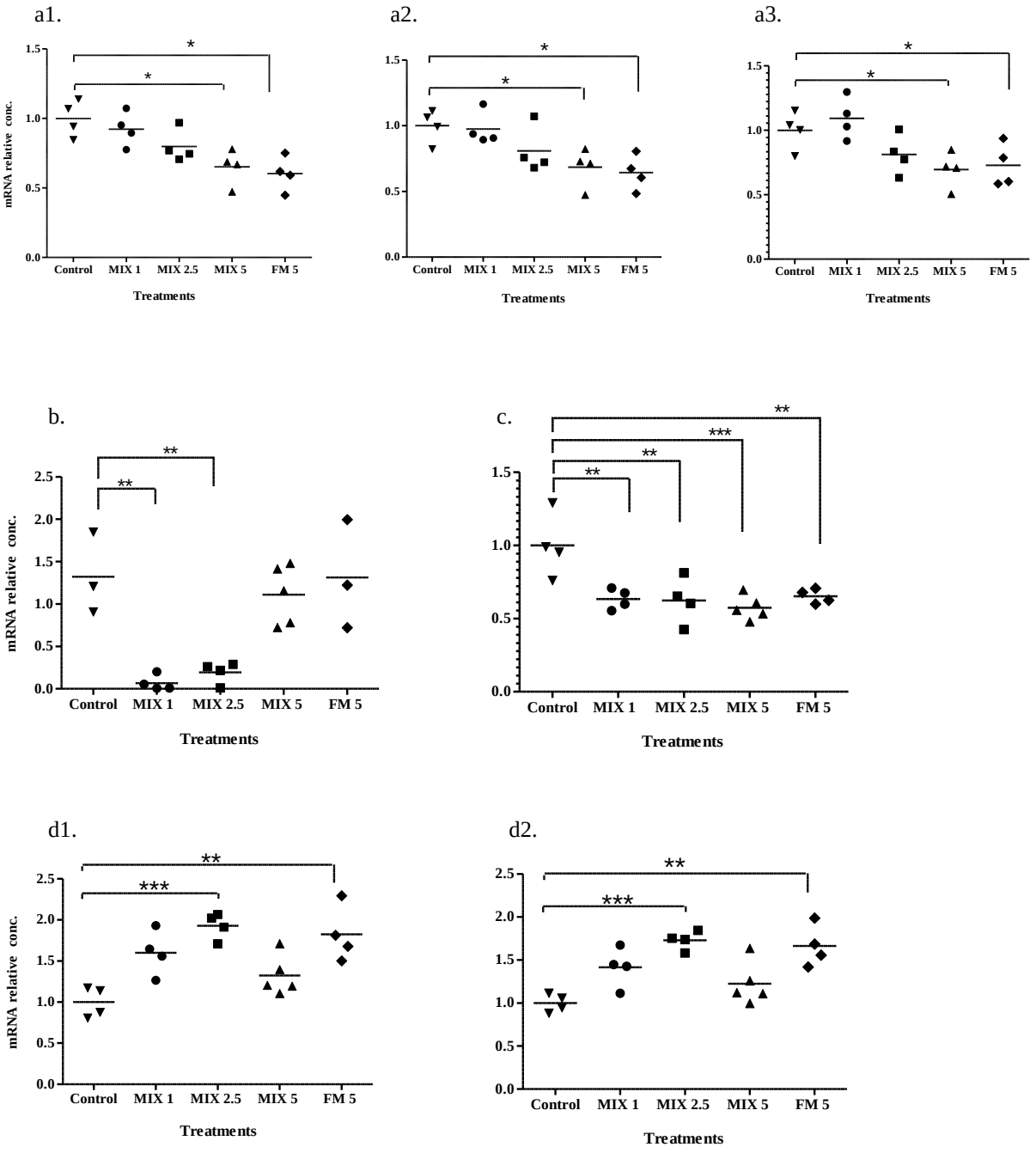

Figure 2. Relative mRNA expression of (a) IL-18; (b) TNF-α and (c) NOD1 and (d) TLR-4 in IPEC-J2 cells after different extract treatments. Each dot represented independent cDNA product generated from mRNA collected from separate wells. Bar for each treatment group expressed mean values. Statistical differences * ($P<0.05$); ** ($P<0.01$); *** ($P<0.001$).

Table 1. mRNA primer sequences and amplicon length (F: Forward, R: Reverse)

Gene symbol	Gene name	Sequence	Amplicon length
B2M	beta-2-microglobulin	F: TGAAGCACGTGACTCTCGAT R: CTCTGTGATGCCGGTTAGTG	70
DEFB1	defensin, beta 1	F: ACCTGTGCCAGGTCTACTAAAAA R: GGTGCCGATCTGTTTCATCT	109
GAPDH	Glyceraldehyde-3-phosphate deh.	F: ACCCAGAAGACTGTGGATGG R: AAGCAGGGATGATGTTCTGG	79
HPRT1	Hypoxanthine phosphoribisyl transferase I	F: ACACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71
IL6(35)	Interleukin 6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	116
IL6(L118)	Interleukin 6	F: CCTCTCCGGACAAAACTGAA R: TCTGCCAGTACCTCCTTGCT	118
IL8 (37)	Interleukin 8	F: GAAGAGAACTGAGAAGCAACAACA R: TTGTGTTGGCATCTTTACTGAGA	99
IL8(36)	Interleukin 8	F: TTGCCAGAGAAATCACAGGA R: TGCATGGGACACTGGAAATA	78
IL18	Interleukin 18	F: CAATTGCATCAGCTTTGTGG R: TCCAGGTCCTCATCGTTTTC	78
IL18 (48)	Interleukin 18	F: CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC	100
IL18 (L124)	Interleukin 18	F: GAAGTGTTCCTGGACATGATGA R: CCACATTCATCCTTTTCTTTCA	124
NOD1	Nucleotide-binding oligomerization domain 1	F: GAGCAAAGCTGTCGTCAACAC R: GGGCATAGCACAGGATGAAC	98
PPIA	Peptidylprolyl isomerase A	F: CAAGACTGAGTGGTTGGATGG R: TGTCCACAGTCAGCAATGGT	138
RPL13A	Ribosomal protein L13a	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	76
TBP	TATA box binding protein	F: ACGTTCGGTTTAGGTTGCAG R: CAGGAACGCTCTGGAGTTCT	96
TLR4(62)	Toll-like receptor 4	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145

TLR4	Toll-like receptor 4	F: TGGTGTCCCAGCACTTCATA R: CAACTTCTGCAGGACGATGA	116
TNF(74)	Tumor necrosis factor alpha	F: CCCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	92
YWHAE	Tyrosine 3-monooxygenase/ Tryptophan 5-monooxygenase activation protein, zeta polypeptide	F: GCTGCTGGTGATGATAAGAAGG R: AGTTAAGGGCCAGACCCAAT	124

11. Conclusions and Perspectives

Introducing alternative approach to manage Post Weaning Diarrhoea (PWD) in pig production continues to be relevant. Controlled methodology with proven efficacy is desirable for the farmer to adopt any new practice.

The present work exhibited that including whey permeate powder in the diet improved conversion rate of feed to body weight gain. Furthermore, our work using an infection model observed reductions of diarrhoea as one of the responses to probiotic feeding. Overall, the results from this study offered two parts in the knowledge of managing gut health around weaning in piglets: first by including whey permeate powder in the diet, second by adding fermented whey permeate inoculated with *W. viridescens.*

The thesis also provided perspectives to lingering challenges. Establishing an infection model remains a challenge as cross-contaminations during pig trials are rampant. A thorough set-up involving separate housing for the control group is critical. Additionally, assessments of the immune response modulations in piglets are necessary to elucidate the probiotic mechanisms in conferring benefits to pigs. Finally, prior to introducing the application of fermented whey permeate to pig farms, optimizing large scale whey permeate fermentation using the selected probiotic and testing product stability and or efficacy in a real farm condition may be required.

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