Managing the developing gut microbiota of growing piglets – novel prebiotic and probiotic strategies

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ABSTRACT

Nursing is a major critical period in the life of piglets. On one hand maternal antibodies are not able to cross the placenta, thus piglets are born without circulating antibodies and consequently lack maternal passive protection. On the other hand, creep-feeding and weaning increase susceptibility to gut disorders, infections and diarrhea. Therefore clarification of the composition and function of the normal gut microbiota of piglets is pivotal as a knowledge base for the design of innovative nutritional strategies based on pre- and probiotics to keep piglets healthy. The objectives of this study were to describe the composition and function of the intestinal microbiota of piglets during the nursing period through creep feeding and weaning, to *in vivo* and *in vitro* evaluate the effect of daidzein on composition and function of intestinal microbiota of nursing piglets in order to evaluate its prebiotic function, and to investigate the probiotic effect of *Lactobacillus sobrius* S1 on composition and function of intestinal microbiota of nursing piglets.

The porcine intestinal microbiota development and diversity, the *in vivo* and *in vitro* evaluation of prebiotic effect of daidzein and the investigation of probiotic effect of *Lactobacillus sobrius* S1 on composition and function of intestinal microbiota of piglets during nursing period through creep feeding and weaning was described using real-time PCR, and PCR analysis of 16S ribosomal RNA gene by denaturing gradient gel electrophoresis (DGGE) and cloning, in combination with analysis of gas production, lactate and VFA yield. The data obtained during the course of the study indicated that 1) Early creep-feeding stabilizes the microbiota of piglets around the weaning period. 2) *Lactobacillus* communities follow a successional change associated with piglet growth and diet shifting. Creep feeding stabilizes the *Lactobacillus* community of weaning piglets. Within the *Lactobacillus* community, some members like *L. reuteri* and *L. amylovorus / L. sobrius* might be permanent colonizers, while *L. delbruckii, L. acidophilus* and *L. crispatus* are more likely to be transient members of the *Lactobacillus* communities in the piglet´s GI tract. 3) Both *in vitro* and *in vivo* evaluations indicated that daidzein has the potential for use as a prebiotic additive in animal feed. 4) *Lactobacillus sobrius* S1 has the potential of promoting beneficial bacteria and inhibiting pathogens.

Keywords: creep feeding, weaning, intestinal microbiota, *Lactobacillus sobrius*, prebiotics, daidzein, probiotics, 16S rRNA, DGGE, cloning, real-time PCR

To my family

谨于此文献给我的家人们

Contents

CHAPTER 1

General Introduction

The objective of this introduction is to provide the background information and the aim for the studies described in the following chapters, which is followed by the outline of this thesis.

1. Gastrointestinal microbiota of nursing piglets

The composition of the microbiota that populates the gastrointestinal tract of newborn piglets develops with age, and is strongly affected by the change of sow's milk to formulated feed until an adult-type pattern is achieved (Inoue et al. 2005; Konstantinov et al. 2006a). The adult-type commensal microbiota produces short chain fatty acids (SCFA) from indigestible carbohydrates by fermentation. These ensure a low pH and are used by other microbes and the host. This process protects the host from pathogens by forming a front line of mucosal defence (Zoetendal et al. 2004). In contrast to adults, the nursing piglet is highly susceptible to enteric diseases because the piglet is immuno-deficient at birth and the commensal microbiota is not yet fully established (Bauer *et al*., 2006a).

It is recognised that microbial fermentation within the GIT is very important for gut health of the pig (Williams et al. 2001). The main products of fermentation include volatile fatty acids (VFA), which are known to play an important role in water (and Na+) absorption, pH control and the inhibition of pathogens. The composition of the diet is crucial in determining the composition and activity of the intestinal microbiota. For example, there is an important difference between the fermentation of carbohydrates and protein. Fermentation of carbohydrates leads to the production of mainly straight-chain VFA (e.g. acetic, propionic and butyric acids) and the use of NH3 and other nitrogenous compounds, which are required for microbial growth. When carbohydrates are in short supply relative to the available protein of non-degradable and endogenous origin, protein will be used as an energy source for fermentation, resulting in the end products NH3, branched chain VFAs, and potentially toxic end products (Williams et al. 2001). Thus, to benefit the host, a balanced commensal microbiota must be organized in a food web that aids in breaking down nutrients, provides the host with energetic substrates, and aid in feeding each other to form a fairly stable community. Weaning, the immediate change of diet, can easily tip the balance of developing commensal microbiota towards one which is associated with disease. Therefore, the development of new strategies for helping piglets towards a rapid development of a balanced and healthy commensal gut microbiota is significant to prevent

the onset of enteric diseases.

2. Managing gastrointestinal microbiota of nursing piglets through the diet

2.1. Creep feed

Creep feeding is a commonly used management for nursing piglets. A solid formulated feed which meets the requirements of nursing piglets is given to suckling piglets in a creep. In most Chinese swine farm, creep feeding starts from piglets at one week of age (Huang, 2003). Intake of a sufficient amount of creep feed during lactation creates a gradual transition at weaning and can reduce the occurrence of post-weaning disorders. However, creep feed consumption during lactation is usually low and is also highly variable among piglets in a litter and between litters (Barnett et al., 1989; Pajor et al., 1991; Kuller et al., 2004b). English (1981) has suggested that adequate creep intake before weaning confers protection against disease. This notion is supported by a recent study showing links between creep intake before weaning and post weaning occurrence of collibacillosis (Carstensen et al. 2005). *E. coli* infection is lower after weaning in piglets which had consumed an optimal amount of creep feed as compared with those consuming no creep feed before weaning. These studies illustrate the importance of creep feed to protect post weaning disorders, however, the direct influence of creep feed on suckling piglets is much less clear.

2.2. Probiotics

The concept of probiotics, which are considered beneficial to the GI tract as an alternative to antibiotics, attracts increasing interests of animal nutritionists and livestock producers. It is defined as a live microbial feed supplement that is beneficial to health (Salminen and others 1998). Feeding trials reported in literature which are aimed at improvements of performance gave significant results in some cases only. There is a high variation between individual animals in the response to probiotics (Simon, 2005). In addition they are rather costly. With probiotic treatment the incidences of diarrhea were reduced significantly in most feeding trials after weaning. For example, the probiotic effect of *L. sobrius* 001T against ETEC K88 has been examined using *in vitro* and *in vivo* approaches. Supplementation of a diet based on fermentable fibre with *L. sobrius* improved the body-weight gain and immunity of weaned pigs orally challenged with ETEC K88 and

reduced ileal ETEC abundance (Konstantinov, 2005; Roselli, 2007).

Although considerable efforts have been made with respect to the efficiency and the mode of action of probiotics, our understanding is still far from complete. The major principle is that probiotics act mainly via modification of intestinal bacterial populations, but also through direct interaction with the host. The probiotic effect could be a result of their positive influence on gut microbiota balance (Metzler et al. 2005).

2.3. Prebiotics

A prebiotic has been defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health (Gibson, 2004). The commonly used prebiotics are fermentable carbohydrates. The addition of fermentable carbohydrates to the diet of weaning piglets is regarded as a comparatively straightforward way to improve microbiota composition and functionality in both the small and large intestines of piglets (Williams et al. 2001; Bauer et al. 2006b). The composition of the bacterial community in the gut of weaning piglets was shown to be affected by the dietary addition of sugarbeet pulp, inulin, lactulose and wheat starch, a diet specifically designed to stimulate the fermentation along the entire GIT (Konstantinov et al. 2003, 2004). Fermentable carbohydrates could enhance colonic microbial stability and diversity, with concomitant stimulation of the growth of *Lactobacillus sobrius*, a newly identified and beneficial member of the porcine commensal microbiota (Konstantinov et al. 2004, 2006b).

Daidzein is a phytoestrogenic compound which is naturally present in soy bean at levels of about 1 to 5 mg/g (Eldridge *et al*., 1983; Wang *et al*., 1994). Many studies in our laboratory have demonstrated that daidzein has anabolic effects on animal metabolism and can affect performance (Han, 2006). They may affect not only the neuroendocrine system, but also the gut microbiota of animals. Daidzein has recently been introduced as a feed additive in farm animal nutrition in China. Comparing to the fermentable carbohydrates, daidzein is not a preference substrate of intestinal microbiota. The underlying mechanism of the effect of daidzein on gut microbiota remains unsolved so far.

The studies in this thesis focus on a number of aspects of microbiota presence and activity in young piglets. These aspects deal with age, as well as with oral administration of either daidzein or *Lactobacillus sobrius* S1. A cultivation-independent 16S ribosomal RNA

(rRNA)-targeted approach has been used to describe the microbiota.

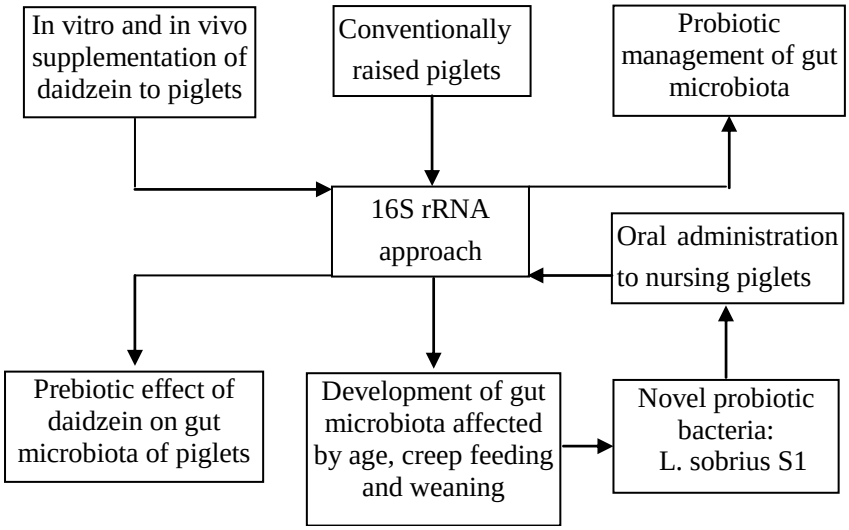

Figure 1.1. Schematic overview of the aspects in this thesis.

3. Outline of this thesis

Chapter 2 provides an extensive review of the research progress on metabolism of isoflavones in the intestinal tract and the research progress of the effect of isoflavonic compounds including metabolites on the physiology, gut microbiology and performance of farm animals in China.

Chapter 3 and 4 mainly focus on molecular characteristics of intestinal microbiota of piglets during the nursing period through creep feeding and weaning. Chapter 3 studies the development of the fecal bacterial community, whereas Chapter 4 focuses on the *Lactobacillus* community specially. Molecular diversity and development of the *Lactobacillus* community in the intestinal tract, as influenced by age, diet and different compartment are studied.

Chapter 5 and 6 are designed for *in vitro* and *in vivo* evaluation of prebiotic effects of daidzein. Chapter 5 investigates the impact of the phytoestrogen daidzein on compositional and functional aspects of the porcine ileal and colonic *Lactobacillus* community during the nursing period through *in vitro* fermentation. Chapter 6 studies the *in vivo* effect of daidzein on the intestinal bacterial communities of piglets by 16S rRNA-based techniques.

Chapter 7 monitors changes in the composition of microbiota in the hindgut of piglets after oral administration of an isolate obtained from piglets used in Chapter 4,

Lactobacillus sobrius S1, using molecular techniques based on 16S rRNA genes.

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CHAPTER 2

Isoflavonic Phytoestrogens – Their Metabolism in the Intestinal Tract and Function in Farm Animals in China

This chapter has in part been published in:

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X Zhang, W Yao and WY Zhu. 2006. Research progress in intestinal bacteria for metabolizing soybean isoflavones. World Chinese Journal of Digestology. 14: 973-978.

Abstract

Isoflavones are a certain class of estrogenic compounds that are often associated with a reduced risk of cancers. The estrogenic activity can be enhanced after metabolization into more active compounds such as equol by gut microorganisms. The direct use of these metabolites has been investigated in laboratory rats and farm animals over the last decade. The present paper reviews the research progress on metabolism of isoflavone in the intestinal tract. This includes the role of intestinal microbiota on its metabolism, isolation of isoflavone-degrading bacteria and the relationship between equol-producing capacity and the composition of individual microbiota, and the research progress of the effect of isoflavonic compounds including metabolites on the physiology, gut microbiology and performance of farm animals in China.

Introduction

Phytoestrogens are plant-derived substances that are able to activate the mammalian estrogen receptors. Most of these plant substances are to some extent similar to the mammalian estrogen either in structure or function. Besides their estrogenic activity phytoestrogens also exert a lot of non-hormonal actions such as assumed cancer protection or antioxidant effects (Beck et al., 2005). Isoflavones are a group of phytoestrogens, and they can be found in many plants, particularly in the legume family. Isoflavones are often associated with a reduced risk of breast-, colon- and prostate cancer from various reviews (Han and Wang, 1994; Han, 1999; Setchell, 1998; Setchell and Cassidy, 1999; Cassidy et al., 2000) and more recently reviewed by Magee and Rowland (2004). It is widely believed that the relatively low incidence of breast and prostate cancer in China, Japan and Korea is mainly associated with the large quantity of consumption of soy products (Adlercreutz, 1990; Messina et al., 1994). Extensive researches have been conducted to investigate the effect of isoflavones on human health. The underlying mechanisms have been studied in animal models and also by in vitro studies (King and Bursill, 1998; Setchell, 1998).

It has been established very well that the conversion of isoflavone is advantageous because of the higher estrogenic potency of their metabolites and also that the intestinal microbiota play a key role on isoflavone metabolism. The discovery of individual differences in equol producing capacity in recent studies focused attention on isolation of isoflavone degrading bacteria, and identification of microorganisms that have equol-producing capacity. The first aim of this paper was to summarize research progress in this regard.

Clinical studies have also demonstrated the potential of isoflavones in clinic nutrition (Setchell and Cassidy, 1999). In China, many isoflavones and their original plant extracts have been used as main components of the traditional Chinese medicine for thousands of years (Han and Shen, 1991). Over the last decade, China has also made extensive efforts to identify and understand the effects of isoflavones on farm animals due to the impact of the successful application of isoflavonic compounds in clinical human medicines. Many studies in our laboratory have demonstrated that isoflavones do have anabolic effects on animal metabolism and can affect performance. They may affect not only the neuroendocrine system, but also the gut microbiota of animals. Of all the isoflabonic phytoestrogens, daidzein has been most extensively studied and has recently been

introduced as a feed additive in farm animal nutrition in China. The second aim of this paper was therefore to summarize research progress in this regard in China.

1 Metabolism of isoflavons in the intestinal tract

1.1 The forms and chemical structure of isoflavones

Isoflavones are a group of phytoestrogens (Figure 2. 1), that can be found in many plants, particularly in the legume family. Soybeans and soy products are the most significant dietary sources of isoflavones (Coward *et al*., 1993; Wang *et al*., 1994). All soybean proteins and foods currently available for human consumption contain significant amounts of the isoflavones. They contain mainly three isoflavones, and each is found in four chemical forms (Figure 2. 2). The aglycones (unconjugated forms) are daidzein, genistein, and glycitein. Each of these isoflavones can be in the form of a glucoside (daidzin, genistin, and glycitin), acetylglucoside and malonylglucoside (Kudou *et al*., 1991).

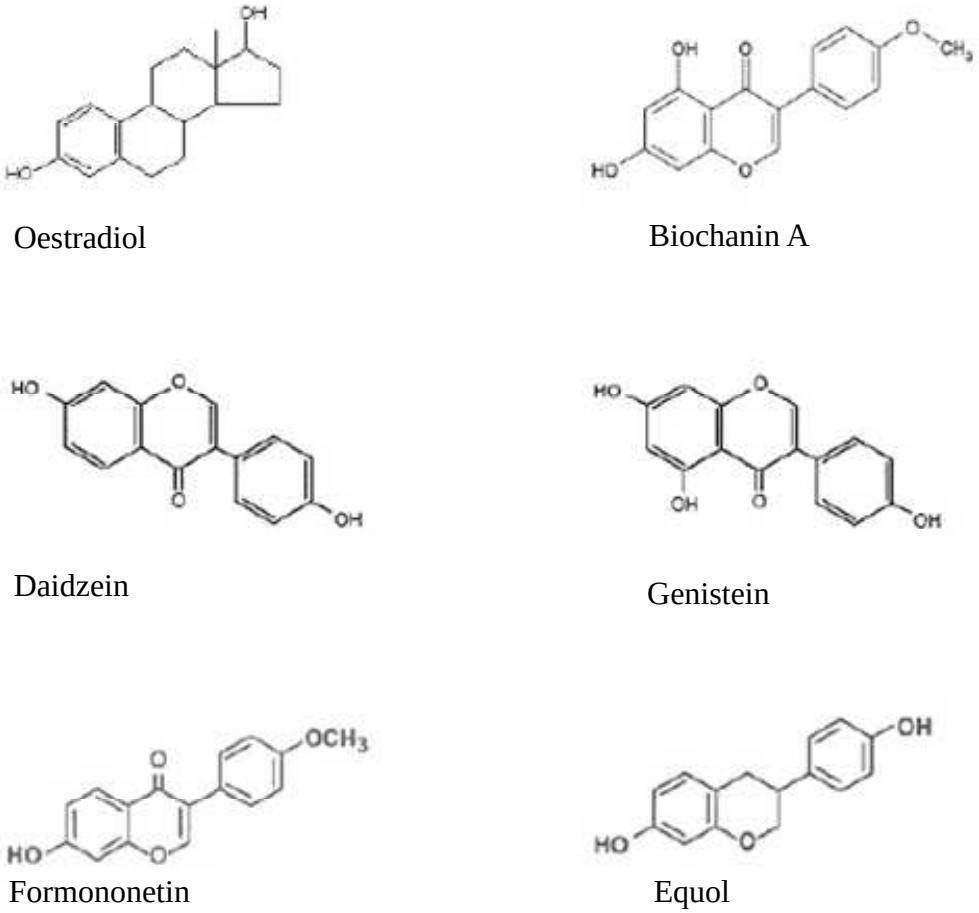
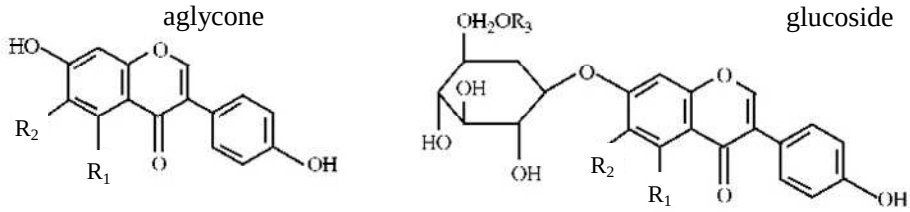


Figure 2. 1. Chemical structures of isoflavonic phytoestrogens.



Class	Isoflavone	R1	R2	R3
aglycones (unconjugated forms)	Daidzein	H	H	-
	Genistein	OH	H	-
	Glycitein	H	OH_3	-
glucoside	Daidzin	H	H	H
	Genistin	OH	H	H
	Glycitin	H	OH_3	H
acetylglucosides	6’’-O-acetyldaidzin	H	H	COCH3
	6’’-O-acetylgenistin	OH	H	COCH3
	6’’-O-acetylglycitin	H	OH_3	COCH3
malonylglucosides	6’’-O-malonyldaidzin	H	H	COCH2COOH
	6’’-O-malonylgenistin	OH	H	COCH2COOH
	6’’-O-malonylglycitin	H	OH_3	COCH2COOH

Figure 2. 2. The four chemical forms of the soy isoflavones. (Kurzer and Xu, 1997; Kudou, *et al*., 1991).

The content of isoflavones in soy-foods shows great diversity. For most of the soy flours and concentrates, isoflavone concentrations are relatively high (0.5–3.0 mg/g), whereas the soymilks and soy infant formulas have relatively low concentrations of isoflavones (0.2–1.0 mg/g). Tofu has significant amounts of isoflavones (0.2–0.5 mg/g), however, the concentrations are highly variable between types and brands of tofu. Soy oils have only traces of isoflavones. Soy sauce also has low isoflavone content. Fermented soybean products have a predominance of the aglycones, because many of the bacteria used in their preparation are capable of hydrolyzing the glycosidic conjugates (Setchell, 1998). These implicate that the daily ingestion of isoflavones varies individually associated with individual diet compositions and sources. Up to now, physiological levels of isoflavones have not been reported in human and animal studies, despite the fact that plenty of investigations have been conducted in last few decades. An early study showed that the daily consumption of 45 mg of isoflavones from 60 g of textured vegetable protein led to a significant increase in menstrual cycle length

(Cassidy et al., 1995). A summary on results of 16 studies shows that the used isoflavone doses varied from 0.12 to 11.9 μmol/kg BW or from 0.9 to 56.5 mg/kg BW (Nielsen *et al*., 2007).

1.2 The metabolic pathways of soy isoflavones

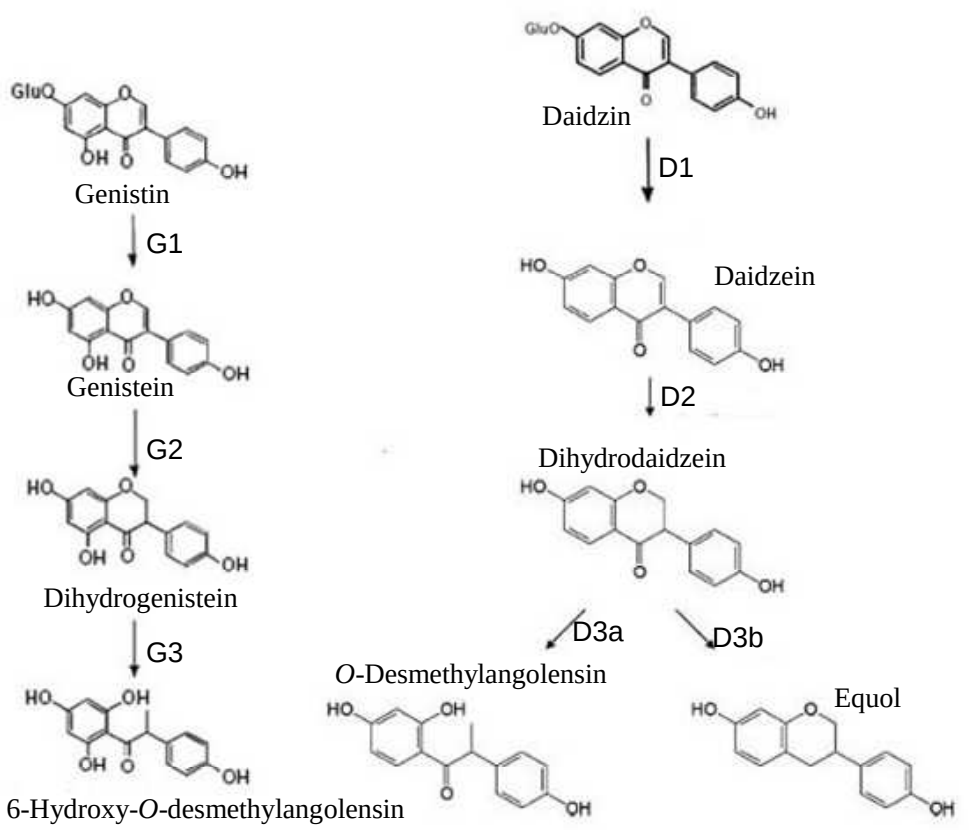


Figure 2. 3. The metabolic pathways of daidzein and genistein in humans (Setchell *et al*., 2002; Setchell *et al*., 1988; Joannou *et al*., 1995). They both include three steps, marked as G1to G3 for genistin and D1 to D3a and D3b for deidzin.

Soy isoflavones are present predominantly as glucosides (daidzin and genistin especially) in most commercially available soy products, with the exception of fermented soy products (Hollman and Katan, 1998). Setchell et al. (2002a) clearly showed that isoflavone glucosides are not absorbed intact across the enterocyte of healthy adults. Their bioavailability requires initial hydrolysis of the sugar moiety by intestinal β-glucosidases before uptake into the peripheral circulation. Their hydrolyzed products, aglycones

(daidzein and genistein mainly), can undergo fermentation by animal and human intestinal bacteria. The metabolic pathways of daidzein and genistein in humans were originally proposed by Setchell & Adlercreutz (1988) and have been expanded by Joannou et al (1995), based on the isoflavone metabolites found in human urine (Figure 2. 3). Daidzein is metabolized into dihydrodaidzein (DHD), which is further metabolized into both equol and O-desmethylangolensin (O-DMA). Genistein is transformed to DHD and is further metabolized to 6'-hydroxy-O-DMA.

The metabolism of isoflavones in animals, including sheep, domestic fowl, laying hens, goats and cows has been well reviewed by Setchell, et al. (2002). In sheep, formononetin and biochanin A are biotransformed by ruminal bacteria to the demethylated intermediates, daidzein and genistein, and then to the estrogenic metabolite equol and the inactive metabolite *p*-ethylphenol, respectively.

It has been well known that the hydrolyzation of isoflavone glycosides can stimulate their absorption, and their further conversion (especial equol-production) can enhance their estrogenic potency *in vivo* (reviewed by Setchell *et al*., 2002b). The binding affinity of equol with estrogenic receptor (ER) is higher than that of daidzein while it is similar to that of genistein, but equol induces transcription more strongly than any other isoflavone, especially with ERα. More equol, 49.7% of equol comparing to 18.7% of daidzein or 4.6% of estradiol, circulates in the free or unbound form in human serum, suggesting more equol is available for receptor occupancy. This implicates that biotransformation of isoflavones is needed to release their potential bioavailability.

1.3 The role of intestinal microbiota in soy isoflavonic metabolism

The crucial importance of the gut microbiota in soy isoflavonic metabolism has been convincingly demonstrated both *in vitro* and *in vivo*. Early studies suggested intestinal microbial enzymes (β-glucosidases) needed for hydrolysis are present in several groups of bacteria including lactobacilli, bifidobacteria and bacteroides (Xu *et al*. 1995). Hur *et al*. (2000) confirmed that daidzin and genistin can be deglycosylated to daidzein and genistein, respectively, by β-glucosidase derived from human intestinal bacteria and pure culture isolates (see below). Day *et al*. (1998) reported that cell-free extracts from human small intestine contain β-glucosidase activity, which can hydrolyze various natural isoflavonic glycosides. Furthermore they purified lactase phlorizin hydrolase (LPH) from

sheep small intestine. This preparation was capable of hydrolyzing a range of isoflavone glycosides. LPH is a membrane-bound, family 1 beta-glycosidase found on the brush border of the mammalian small intestine (Day *et al*., 2000). These results suggest that β-glucosidase present in the gut mucosa is capable of hydrolyzing some, although not all, isoflavonic glycosides in foods. This was also confirmed by a result from germ-free rats, which excreted large quantities of daidzein and genistein in urine after the consumption of intact glucoside isoflavones in soya protein (Rowland *et al*. 1999). These results show that that the β-glucosidase from the gut mucosa and the gut microorganisms both can be involved in the deglycosylation of isoflavonic glycosides. Isoflavonic glycosides may be rapidly hydrolyzed by mucosal β-glucosidase and may be further hydrolysed by bacterial β-glucosidase. This can be a reason that the blood level of genistein and daidzein increases after only 15 min of ingestion, with thereafter a short period of peak concentration of daidzein and genistein (Rowland *et al*. 1999).

For the further conversion of genistein and daidzein, the necessity of intestinal bacteria has been well proven. Chang & Nair (1995) have shown that conversion of daidzein into DHD, benzopyran-4, 7-diol, 3-(4-hydroxyphenyl) and equol by human faecal bacteria occurs and also the conversion of genistein to DHD. Results from antibiotic-treated and germ-free subjects confirmed the role of the intestinal microbiota in isoflavonic aglycone metabolism. Setchell *et al*. (1981) reported reduced excretion of isoflavone metabolites in antibiotic-treated human subjects. The absence of equol and O-DMA in the urine of germ-free rats fed a soy-isoflavone containing diet, and the detection of them in the urine of these germ-free rats colonized with human faecal microbiota provides further evidence that equol and O-desmethylangolensin are products of gut microbiota activity (Bowey *et al*., 2003).

1.4 The isolation and identification of isoflavone-degrading bacteria

Among the gut microbiota, only a few intestinal bacteria have so far been identified, which are able to metabolize isoflavones, and most of them are from the human intestine (Table 2. 1).

Hur *et al*. (2000) isolated two strains of bacteria from the feces of a healthy individual (*E. coli* strains HGH21 and HGH6), which are capable of hydrolyzing the natural isoflavone glycosides daidzin and genistin to their respective aglycones daidzein and genistein.

Under anoxic conditions, strain HGH6 further metabolized daidzein and genistein to DHD and dihydrogenistein (DHG), respectively, but did not further metabolize DHD and DHG. Hur *et al*. (2002) further reported that a human intestinal bacterium, *Clostridium* sp strain HGH 136, could cleave the C-ring of daidzein and DHD to produce O-DMA. The other strain isolated from human feces by Schneider (2000), *Eubacterium ramulus*, was shown to be able to cleave C-ring of both daidzein and genistein (Schoefer *et al*., 2002). Genistein was completely degraded by *E. ramulus* via 6'-hydroxy-O-DMA to 2-(4-hydroxyphenyl)-propionic acid. Daidzein was partially degraded to O-DMA. A strain from the bovine rumen, named as Niu-O16, has the capability of anaerobically converting daidzein and genistein to DHD and DHG, respectively (Wang *et al*., 2005, JB). For equol production, Wang *et al*. (2005) isolated a strain *Eggerthella* sp. Julong 732 with the capability of converting only DHD to the unique metabolite *S*-equol under anaerobic conditions. These series of studies implicate that the biotransformation of the isoflavones daidzin and genistin occurs by sequential action of a group of bacteria. Decroos *et al*. (2005) have confirmed this hypothesis. From human faecal samples, they obtained a mixed bacterial culture that can transform daidzein into equol, and four bacterial species involved were identified by further molecular fingerprinting analysis (denaturing gradient gel electrophoresis, DGGE). Three of them could be brought into pure culture and identified as *Lactobacillus mucosae* EP12, *Enterococcus faecium* EPI1 and *Finegoldia magna* EPI3 and could not produce equol from daidzein in pure culture. This suggests that a combination of some bacterial strains is of crucial importance for the complete degradation of soy isoflavone to equol in humans. In the same report, it was also revealed that colonic fermentation products from poorly digestible carbohydrates, such as hydrogen gas in particularly but also butyrate and propionate, stimulated equol production in this mixed culture. This suggests that there is cross-feeding between soy isoflavone-degrading bacteria and other groups of bacteria, which may be also one of the reasons why there was lack of equol production in pure cultures. Furthermore, recent research with using a combination of two pure cultured bacterial strains, *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16, which have been known to transform DHD to S-equol and daidzein to DHD, respectively, confirms their role (Wang, *et al*., 2007). This anaerobic incubation mixture produced S-equol from daidzein through DHD, and the production of S-equol from daidzein was significantly enhanced compared to that from DHD by

Eggerthella sp. Julong 732 alone.

On the other hand, efforts for getting some pure microbial isolates which can completely convert soy isoflavone glycosides and/or aglycones to the end product equol are still ongoing. For the first time, Minamida, *et al.* (2006) isolated an anaerobic gram-positive rod-shaped strain do03 from rat intestine, which is capable of producing equol from daidzein. Our laboratory isolated two strains zx-5 and zx-7 from porcine feces, which are able to degrade daidzein to equol as well (Zhang, *et al.*, 2007). However, whether the equol-producing capacity of these pure cultured strains can be transferred stably *in vivo* and *in vitro* needs to be further confirmed.

Table 2. 1. Pure culture isolates of intestinal microorganisms with proved activity of converting daidzin and its intermediate metabolites

Metabolic pathways of daidzein		Reported isolates	Sources	References
Daidzin→Daidzein (D1)		*E. coli* HGH21, HGH6	Human feces	Hur, 2000
Daidzein→Dihydrodaidzein (D2)		HGH6, *Lactobacillus* sp. Niu-O16	Human feces bovine rumen	Hur, 2000; Wang. 2005JB
Dihydrodaidzein→Equol (D3b)		*Eggerthella* sp. Julong 732	Human feces	Wang, 2005
Daidzein→O-demethylangolensin (D2+D3a)		*Clostridium* sp. HGH 136 *Eubacterium ramulus*	Human feces Human feces	Hur, 2002; Schneider, 2000
Daidzein→Equol (D2+D3b)	Mixtuere	EP12, EPI1, EPI3 and uncultured *Veillonella* sp. Julong-732 and Niu-O16	Human feces See above	Decroos, 2005 Wang, 2007
	Pure isolates	do03 zx-5 and zx-7	Rat caecum Porcine feces	Minamida, 2006; Zhang, 2007

1.5 Equol production is linked to the intestinal microbiota

The metabolic fate of isoflavones has been shown to be different among individuals in humans and monkeys, due to individual differences in gut microbiota composition (Rafii *et al*., 2003, 2004; Atkinson *et al*., 2004). Daidzein can be metabolized to equol, DHD and O-DMA, and about one third of human individuals can convert dietary daidzein into equol, others into DHD and/or O-DMA. Setchell (2002) described these two distinct populations

as an “equol-producer” or “non-equol producer”, based on a definition, where subjects who have plasma equol concentrations of <40 nmol/L (10 μg/L) are characterized as “nonequol producers”; whereas those with concentrations >83 nmol/L (20 μg/L) are “equol producers”.

Whereas germ-free rats do not excrete equol (Axelson and Setchell 1981), those colonised with human faecal microbiota (HFM) from an “equol-producer” have equol in the urine. In contrast, germ-free rats with HFM from a “nonequol producer” do not have equol in the urine (Bowey *et al*. 2003). Furthermore, the equol-producing capacity of a microbial culture isolated from “equol producer” feces could be transformed to a fecal culture originating from a “nonequol producer” *in vitro* (Decroos *et al*. 2005). These results indicate that eqoul formation is exclusively dependent on intestinal microbiota. Consequently the inability of “nonequol producers” to produce equol is a consequence of the lack of specific gut microbiota. On the other hand, the possibility of human “nonequol producer” subjects to obtain the equol-producing capacity *in vivo* still remains unclear so far, and is the focus of current and future research. The equol-producing strains isolated from human and animal intestine (see above) may benefit “nonequol producer” to get equol-producing capacity.

The separation between “equol-producer” and “nonequol producer” is generally used to describe the equol formation capacity in human. Although the majority of previous results with respect to the equol-producing capacity in animals did not show any individual differences, Yu *et al.* (2007) could demonstrate inter-individual variation in pigs. For the first time, our laboratory evaluated the equol-producing capacity of three litters (A, B, C) of Erhualian pigs *in vitro*, using each litter with a sow and three piglets. Results showed that microbiota of 8 pigs from the total of 12 pigs could transform daidzein into equol. Fecal microbiota of two piglets from litter A, sow and one piglet from litter B, and sow and three piglets from litter C could convert daidzein to equol. DGGE profiles revealed that in each litter, those equol-producing pigs showed high similarities, while they had lower similarities to the microbiota profiles of nonequol-producing pigs (Yu *et al*., 2007).

2 Effect of isoflavones in farm animals in China

2.1 Rumen microbiota and metabolism

Natural isoflavones from plants can be easily metabolized by rumen microorganisms to

various compounds with genistein and daidzein as two of the major intermediate metabolites (Van Soest, 1994). Early research in the 1980's showed that long term consumption of large amounts of legumes could cause detrimental effects on fertility of ruminants, the so called "clover disease", due to the isoflavones released from legumes (reviewed by Van Soest, 1994). Later studies with artificial rumen models showed that formononetin (the isoflavone from red clover) could increase ruminal cellulase activity, but significantly inhibited ruminal protease activity and total dehydrogenase activity (Han and Wang, 1999). In water buffalos fitted with permanent rumen- and intestine fistulas, it was demonstrated that injection of daidzein (500 mg/d, 12d) via a duodenum cannula could increase serum testosterone, rumen bacterial protein, ammonia nitrogen and total volatile fatty acids (VFA), while no apparent effect on the ratio of individual VFAs was observed (Chen *et al*., 1999). Research demonstrated that the extent of metabolism in the rumen varied between individual cows. The level depends on the fluctuation of blood testosterone levels, which could enter the rumen with saliva or via the rumen epithelium (Yang *et al*., 1998). Thus, isoflavonic compounds may affect microbial activity and their metabolism by increasing the blood and rumen testosterone levels.

Cultivation independent approaches were used to assess the effect of daidzein supplementation on microbial composition in the goat rumen. By 16S rRNA gene-targeted PCR-DGGE, Yao *et al*. (2004a) were able to detect shifts in rumen microbiota after the supplementation with daidzein in traditional Chinese-breed goats. Although most of the DGGE bands were common to rumen samples of control and daidzein treated, animals, some bands were enriched while others disappeared after the daidzein supplementation. 16S rRNA gene sequence analysis further showed that most of the predominant clones either enriched or disappeared after daidzein treatment showed highest similarities to environmental sequences that in most cases had been retrieved from rumen samples. The results suggest that daidzein could indeed affect the rumen microbial composition.

The direct effect of daidzein on rumen microbial activity was also demonstrated using *in vitro* techniques (Zhu *et al*., 2002). Our research showed that daidzein at 5 and 10 mg/L could significantly increase the proportion of propionate in total VFA when rumen samples from native goats were used as the inoculum. However, this effect disappeared when the concentration of daidzein was above 20 mg/L. A time-course study with 10 mg/L of daidzein showed that the effect on VFA profiles became evident after one hour

incubation. A similar pattern was observed with mixed rumen or faecal anaerobic fungi in gas production and substrate degradation. Using a pure culture of a rumen anaerobic fungus, *Neocallimastix* sp., daidzein at 10 mg/L and 20 mg/L significantly increased cumulative gas production and dry matter (DM) loss. With mixed rumen fungi, daidzein at 10 mg/L also significantly affected gas production, although no significant difference was observed for DM loss. In the same experiment, however, daidzein at higher concentrations did not show significant effects on rumen microbial fermentation. This may be due to the biphasic response, which shows estrogenic effect at low dosage, while anti-estrogenic effect is observed at high dosage. These results demonstrated for the first time the effect of daidzein on rumen microbial activity *in vitro*. As it is widely reviewed, many isoflavones and their metabolites can be absorbed and then enter in circulatory blood (Detchell, 1998; Detchell and Cassidy, 1999). Thus, it can be reasoned that isoflavones and their metabolites after absorption can also affect the rumen microorganisms in addition to their initial direct effect in the rumen. Nevertheless, clarification of the mechanism underlying the biphasic effect of daidzein on microorganisms *in vitro* requires further studies.

These *in vivo* and *in vitro* results have revealed clear effects of isoflavone compounds on rumen microbial activity and composition. Isoflavone compounds may affect rumen microbial activity and composition by increasing the blood and rumen testosterone levels. Alternatively, entering the rumen via saliva and rumen epithelium after absorption and affecting microbes directly might be the other possible pathway to affect rumen microbial activity and composition.

2.2 Effect on gut microbiota in piglets

Similarly to studies with rumen microorganisms, studies in our laboratory using *in vitro* fermentation approaches demonstrated that daidzein could directly affect intestinal microorganisms of the piglet gut. This was the first report of its kind about isoflavones in monogastric animals (Yao *et al*., 2004b). To investigate the effect of daidzein on the *Lactobacillus* community in the piglet gut, an *in vitro* fermentation was conducted using gut contents as inocula. Digesta from 12 conventionally raised piglets of the same litter (each three slaughtered on 7, 14, 21 and 28 days of age) were used in three different *in vitro* fermentation treatments: (a) medium with 5g/l of glucose and 50 mg/l of daidzein; (b) medium with 5g/l of glucose; (c) medium only. By using *Lactobacillus* specific primers

for amplifying the V1-V3 region of 16S rRNA gene, PCR-DGGE revealed that all samples showed similar changes in the community fingerprints after fermentation; some dominant bands disappeared, while the density of other dominant bands obviously increased, suggesting that some species may be enriched while others may not be able to grow under the culture conditions applied. Although DGGE patterns were different between digesta from different GI compartments either before or after fermentation, no apparent difference was observed between treatments for each digesta sample. However, further studies using dilution PCR for the semi-quantitative detection of lactobacilli demonstrated that daidzein treatment significantly increased the number of lactobacilli in batches inoculated with digesta samples from most of the gut compartments. Thus, daidzein may have the potential for use as a prebiotic substance in animal feed.

2.3 Immune effects

Genistein has been recognized as an inhibitor of tyrosine kinases (Setchell and Cassidy, 1999). *In vitro* studies have demonstrated that high levels of genistein could reduce macrophage and natural killer cell numbers and phagocytosis rates by inhibiting tyrosine kinases (Steele and Brahmi, 1988), and decrease T and B lymphocyte production by inhibiting topoisomerase II (Chang *et al*., 1995). Low levels of genistein, however, could elicit natural killer cell activity (Zhang *et al*., 1999) and antiviral replication (Yura et al., 1993). In pigs challenged with porcine reproductive and respiratory syndrome (PRRS), Greiner *et al*. (2001a) demonstrated that soy genistein could enhance serum PRRS virus elimination, decrease interferon activity in the serum, and increase α_1–acylglycoprotein (AGP). Pig's growth performance was also improved. The authors concluded from these results that soy genistein at 200 to 400 mg/kg can be an orally active immune modulator.

With daidzein, similar experiments were conducted in pigs by the above mentioned authors, but different effects were observed (Greiner *et al*., 2001b). Four supplementary concentrates (0, 200, 400, and 800 mg/kg) were designed by using 93.7% of pure daidzein extract and the analyzed concentrations of daidzein in the experimental diets were 33, 232, 432 and 805 mg/kg due to 28.85% of soy protein concentrate used in basic diet formula. Although daidzein could improve growth performance in periods of high viremia, it did not affect growth during periods in which systemic virus concentrations were minimized. Furthermore, daidzein additions of 200 mg/kg and 400 mg/kg were effective, but not 800

mg/kg. Unlike genistein, dietary daidzein did neither decrease serum PRRS virus concentrations nor AGP activity. Our research with pigs, however, demonstrated that oral administration of daidzein in pregnant sows could affect immune function in the mammary organ as well in the neonate piglet (Zhang *et al*., 1995a). The concentrations of antibody to swine fever vaccine in serum and colostrum were significantly increased by 41% and 44%, respectively (Figure 2. 4). This suggested that both the systemic and mammary gland humoral immune functions in sows were clearly enhanced. The concentration of antibodies in neonate piglets from treated sows markedly increased through colostrum absorption. With daidzein administration, growth hormone (GH) and prolactin (PRL) levels of sow serum and colostrum were strikingly enhanced. GH increased 155% and 54%, and PRL increased 86% and 220%, respectively. Meanwhile, the serum somatostatin (SS) level was apparently lower than that of the control animals. It appears that the immune-regulatory effect of daidzein may be involved in the decrease of the SS level and in the increase in both GH and PRL levels.

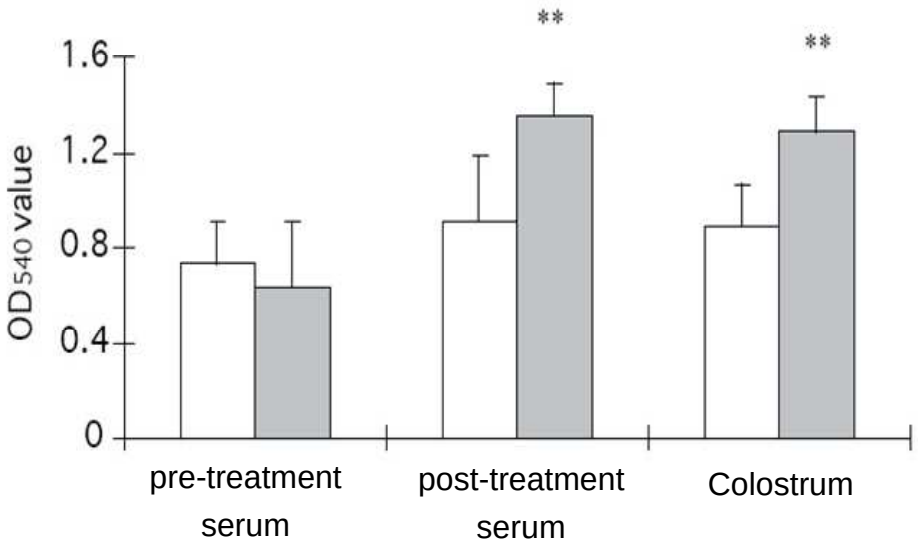


Figure 2. 4. Effect of daidzein on antibody levels of the swine fever vaccine in sow serum and colostrums. Antibody levels in daidzein treatment (■) and controls (□) were expressed by OD values at 540nm as determined by spectrophotometer. Bars are standard errors. **represents significant difference between treatment and its corresponding control. (From Zhang *et al*., 1995a)

Daidzein could also improve the immune function of birds. Daidzein supplementation to diets in male chickens of 7-21 days of age could greatly increase body weight gain and

feed efficiency. The relative organ weight of thymus and bursa, and the T-lymphocyte transformation were all elevated in daidzein treated animals (Gao *et al*., 2000).

In rats, daidzein was shown to increase B and T lymphocyte activity and phagocytosis rate of macrophage cells (Zhang et al., 1997). Our research in mice showed that daidzein and formononetin markedly enhanced the thymus weight and the phagocytosis of peritoneal macrophages. The hemolytic ability of plaque forming cells and the T-lymphocyte percentage in peripheral blood exhibited a significant increase. Either formononetin or daidzein at a dose of 50 μg/mL resulted in more lymphocyte transformation as induced by plant hemoagglutinin (PHA). That transformation was increased by 90% and 210%, respectively (Zhang and Han, 1993). Thus, isoflavonic compounds could affect non-specific, humoral and cellular immune functions and thus could be a plant origin immune modulator.

Isoflavonic compounds also showed anti-aging effects in mice (Wang et al., 1999). Daidzein supplementation to the diets of mice at 18 days of age for one month increased the SOD levels in brain, liver and red blood cells by 22%, 23% and 4%, respectively, while the LPO levels decreased in brain and liver by 36% and 13%, respectively. However, daidzein showed no effects on the SOD and LPO levels in female mice compared to that of control group.

2.4 Effects on mammary gland development and lactation

Formononetin clearly effected the development of mammary glands in ovariectomized mice (Wang *et al*., 1993). Formononetin, which was injected subcutaneously at a daily dose of 0.4 mg/kg for 5 days, significantly increased the relative weight and RNA content of mammary glands. The mammary ducts were well developed, and more secretion granules appeared in acinus. Radio receptor assay analysis further showed that formononetin competed with estradiol for the estradiol receptor of mammary gland cytosol. The maximum binding capacity of estrogen receptors and the prolactin concentration of plasma were markedly elevated after administration of formononetin. It seems that formononetin could enhance the development of mammary glands by directly binding to estrogen receptors of mammary gland cytosol and by promoting the secretion of prolactin from the pituitary.

Similar effects were observed with daidzein, of which the estrogenic activity is about

10-fold higher than that of formononetin. Either administrated orally or subcutaneously, both ovariectomized and conventional young rats showed a marked increase in the development of the mammary gland (Zhang *et al*., 1995).

Radio receptor assays revealed that daidzein not only greatly increased the cytosol binding sites of estradiol in mammary, pituitary and hypothalamus of rats, but also the binding sites of progesterone (Zhang *et al*., 1995). Studies with *in vitro* receptor competitive assays showed an apparent competitive binding affinity of daidzein to the mammary, pituitary and hypothalamus. Thus, the up-regulating effects of daidzein on the receptors of estradiol and progesterone and its positive effects on pituitary GH and PRL secretion may be involved in the mechanisms of daidzein in promoting mammary development.

Further experiments showed that daidzein could significantly affect lactation. Administration of daidzein in rats during late pregnancy could significantly increase milk yield, neonate rat weight gain and mammary gland development pre lactation (Zhang and Han, 1994). With sows, milk yields increased greatly when the late pregnancy sows were fed the diets containing a low dose of pure daidzein extract at 5 mg/kg (Liu *et al*., 1999). On the 10^{th} and 20^{th} day of the nursing period after parturition, the milk yields were 11% (P<0.05, 313.7±17.4 vs. 283.7±10.6 $g \cdot h^{-1}$) and 15% (P<0.05, 371.2±22.2 vs. 333.7±15.1 $g \cdot h^{-1}$) higher than those of the control group. Moreover, the GH, TSH and IGF-1 levels in colostrum were markedly enhanced in daidzein treatments (Table 2. 2).

Table 2. 2. Effect of daidzein on metabolic hormone levels in sow colostrum (n=8)

Group	GH (ng/ml)	Insulin (μU/ml)	TSH (μIU/ml)	IGF-1 (ng/ml)
Control	7.4±2.5	319.7±13.8	8.5±2.8	684.0±95.0
Daidzein	10.6±1.4*	305.15±11.6	12.1±3.7*	897.0±115.0*

Values with * significantly differ from the corresponding control values (P<0.05)

It is generally recognized that dose and duration of intake are two of the important factors that influence the biological effects of isoflavones (Setchell and Cassidy, 1999). The

timing of administration may be another important factor. When daidzein was added to the diet of sows during the lactation period, a different pattern was observed compared to the above-mentioned study conducted on rats. As expected, the milk yield in daidzein treatments was notably higher than that of control animals (P<0.01) at the 5th day after parturition, and maintained stable until day 20 after parturition (Liu *et al*., 1997). Twenty days after parturition, however, the milk yields decreased in daidzein-treated animals as compared with the control. This may be due to a biphasic effect of daidzein. As it is well known, PRL plays an important role in the initiation and maintenance of lactation though many hormones that are involved in regulating the lactation of sows. At the initial stage of lactation when the mammary gland is not completely developed, minute dosage of daidzein acts as a weak estrogen and enhances prolactin levels, consequently increasing the milk yield. As sows ingested daidzein from the diet daily, the accumulated estrogenic effects became greater, the prolactin levels decreased, and even estrus occurred in some sows. Thus, the estrogenic effects of isoflavones can be different depending on the endogenous level of estrogens in the animal.

2.5 Growth

Although widely investigated, the effect of isoflavonic phytoestrogens on growth varied between experiments and between animals. With conventional weaning piglets, soy daidzein or soy genistein did not have significant effects on weight gain and feed intake (Greiner et al., 2001), though daidzein could slightly improve the gain/feed ratio. After challenge with PRRS virus, however, daidzein at 200 or 400 mg/kg is a weak enhancer of body growth and genistein at 200 or 400 mg/kg showed a significant effect on growth performance (see also above in the section on Immune Effects). This may suggest that isoflavonic phytoestrogens may primarily work as immune-modulators rather than conventional growth promoters.

In China, investigations showed positive effects of daidzein on animal growth performance. In studies with growing castrated male pigs, daidzein supplemented to diets at 5 mg/kg significantly increased weight gain by 59% (P<0.01), and the blood IGF-1 and testosterone levels were elevated by 51% and 18%, respectively (Guo *et al*., 2002).

With Redbro male broilers, crude isoflavonic extracts added to the diets significantly increased the serum testosterone levels, while serum uric acids and abdominal fat were

decreased (Wang *et al*., 1994). Daidzein supplemented to the diets at 3 mg/kg significantly enhanced the daily weight gain by 10%, breast muscle and hind leg muscle weight by 6% and 7%, respectively. The ratio of RNA to DNA of muscle cells was greater in daidzein treated animals than that of control group. This suggests that daidzein could promote the muscle protein accretion of male broilers. With female broilers, however, the same dose of daidzein did not produce clear effects on daily body weight gain or serum estrogen levels.

A similar effect was found in growing male rats (Wang *et al*., 1995). Compared to the control group, dietary daidzein supplementation of 3 mg/kg increased daily weight gain and feed intake by 15% ($P<0.01$) and 18% ($P<0.05$), the weights of carcass, gastrocnemius muscle and femur bone increased by 17% ($P<0.05$), 9% and 14% ($P<0.05$), respectively. The femur bone density and blood calcium concentration increased, while alkaline phosphatase activity was lowered by 15% ($P<0.05$). The blood concentration of IGF-1 and testosterone were elevated by 37% ($P<0.05\%$) and 17% ($P<0.05$), whereas estradiol level was reduced by 24% ($P<0.01$). These results suggest that daidzein could improve bone metabolism and growth performance with the effect possibly related with the secretion of the related hormones in growing male rats (Guo *et al*., 2001).

While the effects of daidzein on animal growth in different experiments are not very unanimous, it appears that the effectiveness on growth is more evident for male animals. This is not surprising when considering that daidzein has estrogenic activity. This may also remind us that animal gender should be considered for experimental design. With immune challenged animals, the isoflavonic phytoestrogens seem more effective than with conventional healthy animals. In China, the production environment is less well controlled compared to that in the European Union. The animals would be exposed to many diseases. Indeed, many of our studies demonstrated the positive effect of isoflavonic phytoestrogens on animal growth. Although solid evidence is still lacking, it may be speculated that environmental factors such as disease exposure and other stresses to the animal, can be involved in the amplitude of the isoflavonic effectiveness on growth.

Limited research also showed that daidzein had effects on the fetus growth. The doses of five and 100 mg/kg of daidzein supplemented to pregnant sows and rats respectively, could greatly increase the offspring’s birth weight (Zhang *et al*., 1993; Liu et al., 1996). Nevertheless, more research is needed to investigate the mechanism.

2.6 Egg laying performance of birds

Recent studies have demonstrated that isoflavonic phytoestrogens could improve egg-laying performance in birds. Experiments with 242 and 330 day old layers showed that daidzein supplemented to the diets at 3 mg/kg significantly increased the laying rate of old laying hens. In addition, average egg weight and feed efficiency increased to some extent (Liu *et al*., 1998). This effect was also observed with 174 day old first time laying hens (Meng *et al*., 2001, 2002). Daidzein supplementation significantly increased the egg laying rate and feed efficiency by 9% and 7% in the early laying period, 11% and 14% during the high laying period, and 14% and 17% during the late laying period, respectively.

Similar effects were observed with laying ducks and quails. Daidzein supplementation significantly increased the egg laying rate by 6% in laying Shaoxing ducks, with an overall increase in egg yield increasing by 8% compared to that of the control group during an experimental period of 30 days (Zhou *et al*., 2002). Daidzein treatment markedly prevented body weight loss of Shaoxing ducks and increased ovary weight compared with the control. The number of large follicles, however, did not change. Daidzein supplementation at 3 mg/kg significantly increased laying rates in quails, with values increasing by 7% ($P<0.05$) in the early and in the middle stage, and 10% ($P<0.01$) in the late stage as compared to the control (Wang *et al*., 1999).

Research has also shown that dietary daidzein could increase the feed intake of egg laying hens (Zou *et al*., 2003). By using a Feed Intake Data Acquiring System to monitor the behaviour of the laying hens, Zou *et al*. (2003) were able to demonstrate that supplementation with daidzein at 6 mg/kg increased feed intake during high and late laying periods by 18% and 20% compared to the control, respectively. Meal frequency in a day also significantly increased. Obviously, the increase in feed intake could contribute to the improvement in laying performance of laying hens.

In the meantime, daidzein supplementation could affect the endocrine functions. Daidzein at 3 mg/kg significantly increased blood T3 and progesterone levels of 330 days old laying hens (Meng *et al*., 2001). In Shaoxing ducks, daidzein supplementation at 3 mg/kg significantly increased serum levels of GH, but not IGF-1, while 5 mg/kg daidzein supplementation significantly increased both GH and IGF-1, suggesting a dose-dependent effect (Zhou *et al*., 2002). With laying quails, serum T3 levels in early, middle and late

laying stages were all significantly elevated. Zou *et al*. (2003) showed that while daidzein supplementation increased the feed intake, it also increased insulin and 17 β–estradiol levels in the blood.

Interestingly, the daidzein effect could be different depending on the laying stage (Ke *et al*., 2002; Meng et al., 2001). With 35 day old quails, daidzein supplementation at 3 mg/kg could greatly increased the laying rate and serum T3 level by 7% and 23% as compared to the control. A similar effect was observed with 7 months old quails with daidzein at the same dosage. However, daidzein supplementation at 6 mg/kg led to a decrease in laying rate of the 7 months old quails, while a significant increase was found for 12 months old laying quails. Thus, the doses of daidzein supplementation should be applied in relation to the laying stage of the birds. Similarly, the effect of daidzein on blood 17β-estradiol was also related with the laying stage. The blood 17β-estradiol level of laying hens significantly increased compared with the control, but varied with different laying stages, with higher levels during the late laying stage (Meng *et al*., 2001). For laying quails, the daidzein effect was different, as the blood estradiol levels increased by 63% ($P<0.01$) in the early laying stage, but decreased by 17% in the late laying stage as compared with the control, suggesting a biphasic effect of daidzein in regulation of estradiol levels.

Dietary daidzein could also affect egg content of cholesterol and its oxide (Yin *et al*., 2004). Daidzein supplemented at 40 mg/kg could significantly decrease egg cholesterol content by 19% and egg yolk cholesterol concentration by 11% as compared to the control. While supplementation at 10 and 20 mg/kg showed a similar effect, this effect was not observed with 5 mg/kg of supplementation of pure daidzein extract. The same study also demonstrated that daidzein could also inhibit the formation of cholesterol oxides in cooked egg yolk, with 7-keto cholesterol and total cholesterol oxides content reduced by 27% and 35% respectively, compared to the control. Further studies showed that both daidzein and dietary tea polyphenols exhibited antioxidant effects on laying hens (Yin *et al*., 2003). Lipid peroxidation (LPO) contents in egg yolk, liver and plasma significantly decreased with daidzein or tea polyphenols treatment (both at 40 mg/kg), while the superoxide D~mutase (SOD) and glutathione-peroxidase (GSH-Px) levels remained relatively stable. Thus, dietary tea polyphenols and daidzein could directly improve the bird’s antioxidant levels irrespective of SOD and GSH-Px activities.

The results suggested that dietary isoflavonoids at a proper dosage could have beneficial

effects on laying performance in birds, probably mediated by the regulation of endocrine functions. In addition, the resultant eggs from isoflavonoid supplemented birds could be beneficial to human health.

3 Conclusions

Isoflavonic phytoestrogens can exhibit weak estrogenic activity on reproduction. They can also promote male animal growth, and induce female mammary development and lactation, and improve laying performance of laying birds. These effects were usually coupled with their influence on immunity and metabolic hormones. Thus, the observed effects of isoflavonic phytoestrogens may be mediated by their modulation of immunity and endocrine. Furthermore, environmental factors that can challenge animal immune status could affect the extent to which isoflavonic phytoestrogens influence animal performance.

Intestinal microorganisms play an important role in isoflavone metabolism in the animal gut and consequently influence the metabolic fate of the isoflavones. Humans can be divided into two groups, “equol-producer” and “nonequol-producer”. This may be one of reasons of the disagreement of isoflavone treatments. In return, the isoflavonic phytoestrogens or their metabolites could affect gut microbial activity. This effect can be caused by the circulating isoflavonic metabolites once absorbed by the animal body. However, the *in vitro* effect on microbial activity may also suggest a direct relationship between isoflavonic phytoestrogens and gut microorganisms. Prebiotic effects of isoflavonic phytoestrogens may be involved in this relationship. Clearly, researches have suggested that isoflavonic phytoestrogens have great potential for use in animal feed supplementation in the future.

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CHAPTER 3

Diversity of the Fecal Bacterial Community of Newborn Diarrhea Piglets as Revealed by 16S rRNA Gene-targeted Techniques

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Abstract

PCR and denaturing gradient gel electrophoresis (DGGE) were used to monitor the development and diversity of the fecal bacterial community of 5 newborn diarrhea piglets, which were observed with yellow soft feces at 2 days of age. A clone library was created from 16S rRNA gene fragments amplified from fecal samples of 3 piglets of 42 days of age. From the library, clones that matched predominant bands in corresponding DGGE fingerprints were sequenced and subjected to an online similarity search. Results showed that DGGE profiles of 5 piglets changed from simple (day 2) to complex (day 10), and then from simple (day 16) to complex (day 27) again, and finally remained relatively stable and diverse after weaning. DGGE profiles from day 2 and 16 fecal samples were highly simple and similar, with *E. coli* as the most predominant band. DGGE profiles from day 10 fecal samples were more complex, and the band corresponding to *E. coli* was still present, albeit not predominantly. DGGE profiles from day 35 and 42 fecal samples became complex again, while their predominant bands remained similar and stable. 16S rRNA gene sequence analysis revealed that the 23 clones from the library generated from fecal samples of day 42 health piglets were most closely related to species of *Enterococcus*, *Streptococcus*, *Clostridium*, *Peptostreptococcus, Lactobacillus* and *Bacillus*.

Key words: Denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene sequence analysis, Shannon index, Piglet, Fecal bacterial community

Introduction

The composition of the microbiota that populates the gastrointestinal tract of newborn piglets develops with age, and is strongly affected by the conversion from sow's milk to formulated feed at weaning. This commensal gastrointestinal microbiota plays a fundamental role in host health and disease. Our understanding of its composition and dynamics, however, is still far from complete (Konstantinov *et al*, 2006). Because of the insensitivity of cultivation, molecular fingerprinting methods (such as Denaturing Gradient Gel Electrophoresis, DGGE) and sequence analysis of the 16S ribosomal RNA (rRNA) and the corresponding gene have been increasingly used in revealing mammalian gastrointestinal microbiota (Simpson *et al*., 1999; Zoetendal *et al*., 1998 and 2004). Using DGGE and 16S rRNA gene sequencing technology, Konstantinov and Zhu (2003) investigated the changes of the composition of fecal microbiota of piglets and the effects of selected dietary interventions during the weaning period. In the first week after weaning the composition of fecal microbiota of piglets changes quickly. Fermentable carbohydrates (fructooligosaccharides and sugar beet pulp) can selectively enhance the growth of certain bacteria, and accelerate the stabilization of fecal microbiota (Zhu *et al*., 2003a and 2003b; Konstantinov *et al*., 2003).

In Chinese swine production, a commonly used feeding strategy includes suckling of piglets with sow's milk only in the first week, and then sow's milk with creep feed in weeks 2 or 3. Weaning occurs at 21 or 28 days of age and solely feeding of a solid diet afterwards (Huang 2003). This feeding strategy, early creep feeding and weaning can greatly increase the reproductive productivity of sows. However, it also puts piglets at high risk of diarrhea with a severe growth check and even the death of piglets. This causes large economic losses in the pig industry. Comparing to the diarrhea occurrence of non-creep-fed piglets, where diarrhea mostly develops in the first 2 weeks after weaning (so-called post-weaning diarrhea syndrome) (Lallès *et al*, 2004), the diarrhea in creep-fed piglets mostly occurs before weaning. Mathew (1994) observed an increase of hemolytic *E. coli* in creep-fed piglets at 19 days of age. The aim of this study was to investigate the effect of creep feeding on the succession of fecal microbiota and the occurrence of diarrhea in piglets, using DGGE fingerprinting, and

cloning and sequence analysis of PCR-amplified 16S rRNA gene fragments.

1 Materials and Methods

1.1 Animals, feeding strategy and sampling

Five newborn diarrhea piglets of one litter (P1 – P5) with yellow soft feces at 2 days of age were selected and ear-marked for monitoring the development and diversity of the fecal bacterial community from birth to 42 days of age. Creep feeding was started at 7 days of age. Piglets were weaned at 28 days of age. The fresh feces of piglets were sampled from the rectum at 2, 10, 16, 27, 35 and 42 days of age, and kept at -20°C until further processing.

1.2 DNA isolation and PCR amplification

The total DNA of fecal samples was extracted from 300 mg of feces using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad. CA). Electrophoresis in 1.2% (wt/vol) agarose gels containing ethidium bromide was used to check the amount of DNA visually.

To investigate the fecal bacterial community by DGGE, PCR with primers U0968f-GC and L1401r was performed as described previously (Zhu *et al*., 2003b).

1.3 DGGE of PCR amplicons and analysis of the DGGE gels

PCR products generated with primers U0968f-GC and L1401r (Nubel *et al*, 1996) were separated by DGGE according to the specification of Muyzer *et al*. (1993), using the Dcode system (Bio-Rad Laboratories, Hercules, CA). The gels were poured from the bottom by using a gradient maker and a pump (Econopump; Bio-Rad) set at a speed of 4.5ml/min, and gradients of 30% to 60% were used for the separation of the amplicons. Electrophoresis was performed for 16h at 85 V in a 0.5× TAE buffer at a constant temperature of 60°C.

All DGGE gels were scanned at 400 dpi. The Pearson similarity and the Shannon index of diversity (Shannon, 1963) of the DGGE profiles were calculated based on image analyses of gels by using the Molecular Analyst software (version 1.12, BioRad).

1.4 Cloning and sequence analysis of 16S rRNA genes

A clone library was created from almost complete 16S rRNA genes amplified from fecal samples of 3 piglets at 42 days of age. PCR amplicons obtained with primers 8f and 1510r

(Lane, 1991) were purified using the Qiaquick PCR purification kit (Westburg, Leusden, the Netherlands) according to the manufacturer's instructions. Purified PCR products were further cloned in *E. coli* JM109 cells using the Promega pGEM-T vector system (Promega, Madison, Wis.). To confirm the size of inserts, PCR with pGEM-T specific primers T7 and Sp6 (Zoetendal *et al*., 1998) was performed on lysates of ampicillin-resistant transformants. Amplicons of the correct size were subjected to restriction fragment length polymorphism analysis by using restriction enzyme MspI. Plasmids containing a unique insert of the appropriate size were compared with the original samples on DGGE gels. Plasmids corresponding to predominant bands of the DGGE profiles of fecal samples were purified by using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany), and were subjected to DNA sequence analysis.

Sequencing reactions were performed with the Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, United Kingdom) according to the manufacturer's specifications by using the primer T7 end-labeled with IRD-800. Sequences were automatically analyzed on a LI-COR DNA sequencer 4000L (Lincoln, NE) and corrected manually.

1.5 Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences from the library created from fecal samples of 3 piglets at 42 days of age were subjected to an online similarity search, and then submitted to the GenBank database. The accession numbers of the submitted partial sequences of piglet P1 were as follows: XSHA39 (AY601712), XSHA12 (AY601713), XSHA24 (AY601714), XSHA8 (AY601715), XSHA11 (AY601716), XSHA15 (AY601717), XSHA34 (AY601718), XSHA35 (AY601719), and XSHA5 (AY601720).

2 Results

2.1 The succession of the fecal bacterial community of diarrhea piglets associated with age and shifting of the feeding strategy

Five newborn diarrhea piglets were from the same litter, with normal initial body weight and healthy suckling behavior. The yellow soft feces could be squeezed out of the anus while sampling at 2 days of age. There were no remains of yellow feces, and samples had to be taken from the rectum of piglets by using a sterile cotton rod at 10 days of age. At 16 days of age, piglets P2 and P5 were observed with yellow soft feces

again. At 27, 35, and 42 days of age, no yellow soft feces were observed for any of the 5 piglets included in this study.

DGGE profiles of all 5 piglets changed from simple (day 2) to complex (day 10), and then from simple (day 16) to complex (day 27) again, finally remaining fairly stable and diverse after weaning (Figure 3. 1). All DGGE profiles from day 2 feces were highly similar and shared the same predominant band, which corresponded to a clone migrating to the same position in the DGGE gel with *E.coli* JM109. Partial sequencing confirmed the identity as *E.coli* (data not shown). This suggests that *E. coli* constituted a predominant population in the intestine of the newborn diarrhea piglets. DGGE profiles from day 10 feces, i.e. 3 days after initiating creep feeding, were more complex with more than 10 bands. The band corresponding to *E. coli* was still visible, but not as the predominant band. DGGE profiles from day 16 were again highly similar with those from day 2, with *E. coli* as the predominant band again. DGGE profiles of samples taken after weaning (day 35 and 42) became complex again, while their predominant bands remained similar and stable. When compared to the DGGE profiles obtained from samples taken before weaning, the DGGE profiles after weaning showed obvious differences.

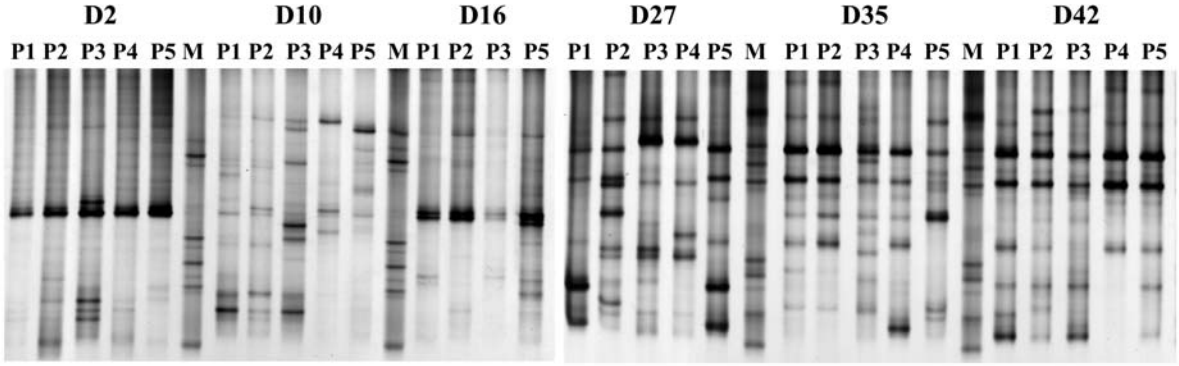

Figure 3.1. Bacterial DGGE profiles generated from V6-V8 16S rRNA gene fragments amplified from piglets fecal samples. Dx: the age of piglet; Px: the number of piglet; M: the marker.

Pearson similarity coefficients were calculated from DGGE profiles to assess the dynamics of fecal bacterial community composition of piglets at different age (Figure 3. 2). The profiles obtained from samples taken on day 2 and day 16 showed highest similarity with an average similarity coefficient of 71.9%. The similarity coefficients of the DGGE profiles of day 10 with those of day 2, day 16 and day 27 were 24.7%, 18.5%

and 29.9%, respectively. Comparing the profiles of day 10 to those of day 35 and 42, the similarity coefficients were higher, with values of 31.6%, and 44.3%, respectively. Comparison of DGGE profiles obtained from samples taken directly before weaning (day 27) with those after weaning (day 35 and 42) revealed similarity coefficients of 26.3% and 39.2%, respectively. These values for comparison of profiles of day 27 with day 35 and 42 were higher than those of day 10 with day 2 and day 16, although the difference was not significant. Finally, a much higher similarity coefficient (62.3%) was observed between the DGGE profiles of day 35 and day 42, indicating a stabilization of the fecal microbiota two weeks after weaning.

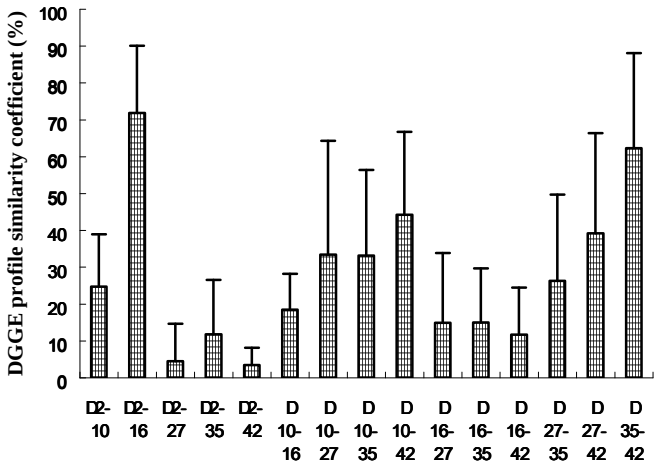

Figure 3.2. Pearson similarity coefficients for the pairwise comparison of fecal bacterial DGGE profiles of piglets at different age. Dx: the age of piglet. Error bars indicate standard deviation.

2.2 Dynamics of fecal bacterial community diversity measured by the Shannon index

For samples taken from day 2 to day 42, the Shannon indices were 1.38±0.55, 2.12±0.24, 1.97±0.26, 2.19±0.29, 2.20±0.17 and 2.14±0.20, respectively (Figure 3. 3). During this 42 days period, the diversity was comparable for days 10 and from day 27, whereas samples taken at day 2 and 16 had a lower diversity.

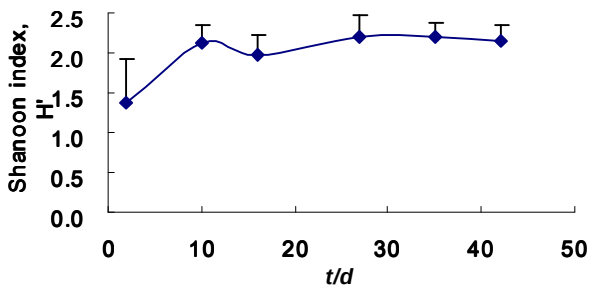

Figure 3.3. Shannon diversity indices of piglet fecal bacterial community. Error bars indicate standard deviation.

2.3 Identification of the dominant bands on DGGE profiles using 16S rRNA gene sequencing.

To identify the composition of the fecal bacterial community of piglets, a clone library was prepared from PCR-amplified almost complete 16S rRNA genes of 42-day fecal samples of piglet 1, 2 and 5. From the library, 23 clones of which the V6-V8 regions matched dominant bands on the DGGE gel were partially sequenced, and sequences were subjected to an online similarity search. Three of these 23 clones had their sequences most closely related to *Lactobacillus* spp. Twelve clones were closest to *Clostridium*, four to *Enterococcus*, two to *Streptococcus*, and the remaining two clones were most closely related to species of *Peptostreptococcus* and *Bacillus*, respectively (Figure 3. 4).

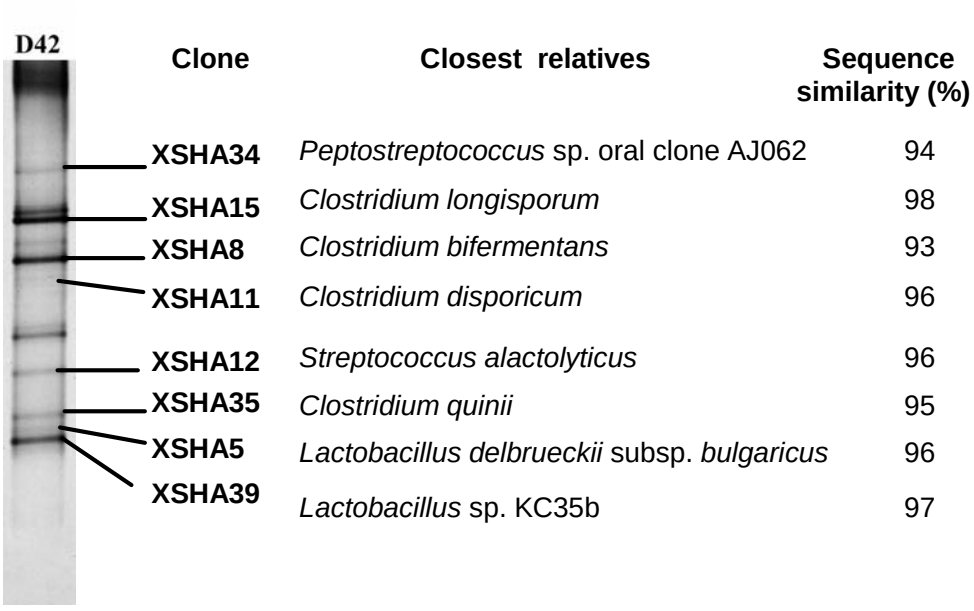

Figure 3.4. Identification of dominant bands in the DGGE pattern of the V6 to V8 regions of rRNA gene amplicons obtained from fecal samples from piglet 1.

3. Discussion

Just before birth the guts of all animals are free of bacteria. The gut microbiota develops very rapidly after birth, and microbes present in the vagina of the mother are a major source of primary inoculum to the piglets. The large populations of microbes which are present in the feces of the mother and within the new environment are additional sources (Bauer *et al*., 2006a). *E.coli* and *Streptococus* spp. may be the first colonizers. Within 2 hours of birth these two bacterial species can be detected in the feces of newborn piglets, and by 5-6 hours their numbers are very high (10^9 and 10^{10}/g feces, respectively) (Ewing and Cole, 1994). In the pig, maternal antibodies are not able to cross the placenta and piglets are born without circulating antibodies, and consequently lack maternal passive protection (Bauer *et al*., 2006b). Some newborns suffer from neonatal *E. coli* diarrhea (the yellow or white diarrhea). Most of diarrhea newborns can recover quickly after suckling maternal colostrum, which is very high in bacteriostatic and anti-adhesive factors (e.g. IgA) specifically directed against pathogenic *E. coli* (Ewing and Cole, 1994). As a result the predominance of lactobacilli is further enhanced. This predominance is fairly stable during the period where newborns are suckled by their mothers (Mathew *et al*., 1998). It is suggested that the higher the L:C-ratio (lactobacilli to coliform ratio) is, the better is the microbial contribution to growth performance of the host (Ewing and Cole, 1994). The rapid changes of environmental conditions that piglets experience during the weaning transition, including separation from the mother, diet change, transportation and change in housing, can induce a decrease of the abundance of lactobacilli and an increased population sizes of coliforms (Tannock and Svage, 1974). It has to be noted that the above-mentioned studies are based on cultivation, which may have been biased against the detection of strict anaerobes. Hence the function of the gut microbiota and the mechanism of neonate diarrhea of piglets are still not fully understood. The aim of this study was therefore to describe the succession of fecal microbiota of neonate diarrhea piglets using cultivation-independent approaches.

Although DGGE fingerprinting of PCR-amplified fragments of the 16S rRNA gene has its own restrictions, it is now well accepted as a fast and reliable method to provide insight into gut microbiota without the limitation of cultivability. In this study the DGGE profiles retrieved from piglets' feces visually showed the succession of fecal microbiota of 5 newborn diarrhea piglets in time. A band corresponding to *E. coli* was

observed in all DGGE profiles, irrespective of the diarrhea status of the piglets. Nevertheless, this band was predominant in samples taken at day 2 and 16 of age of piglets associated with diarrhea. These results suggest that *E. coli* is a normal bacterial inhabitant of earlier colonized piglets. Its rapid multiplication may directly relate to the occurrence of diarrhea in piglets in this study. Schulman (1973) reported similar changes by cultivation (Ewing and Cole, 1994). At day 10 of age (3 days after creep feeding started), DGGE profiles became obviously complex with more than 10 clear bands. Some of these bands still appeared in the profile of weaned piglets, while others disappeared after weaning. This and the high similarity coefficients between samples from day 10 and after weaning (day35 and 42) suggested that creep feed can steer the development and composition of intestinal microbiota of piglets. Diet is one of main factors to influence unestablished microbiota of nursing piglets (Konstantinov *et al.*, 2006). The gut of piglets of 7 days of age is not adjusted to solid feeds, and lacks several enzymes needed for digestion of solid diets. As a result chyme from creep-fed piglets contains more nutrients and thus, creep feed may accelerate the growth of *E. coli* and the appearance of diarrhea. Mathew (1994) observed an increase of hemolytic *E. coli* of creep-fed piglets at 19 days of age. It has previously been postulated that hypersensitivity to feed antigens may also contribute to a decreased absorption of nutrients and thus the induction of diarrhea. Barnett *et al.* (1989) observed increased antibody titers in the blood of weaned pigs and confirmed that feed antigens can induce an immune reaction in creep-fed pigs. Thus, although there is no conclusive evidence available, the observations summarized above constitute several potential reasons that *E. coli* became the predominant band of DGGE profiles again at 16 days of age. Comparative analysis of DGGE profiles also indicated changes in fecal community composition before and after weaning, in line with other studies (Konstantinov *et al.*, 2006). Nevertheless, these differences were not as dramatic as those observed when comparing profiles of samples taken before and after creep-feeding. This indicated that creep feeding is a big challenge for suckling piglets, inducing an unbalance of the yet-unstable developing bacterial community. On the other hand, our results suggest that creep feeding tends to reduce the effect of the stress of weaning on the fecal bacterial community. The Shannon indices before and after weaning didn’t show large differences, which was in agreement with the observation of Konstantinov (2006).

Creep feeding could positively affect microbiota development by stabilization of fecal bacterial community composition before and after weaning, and could decrease the diarrhea appearance caused by weaning. This is in agreement with observations reported from previous and current studies. Intake of sufficient amounts of creep feed during lactation creates a more gradual transition at weaning and can reduce the occurrence of post-weaning disorders. However, creep feed consumption during lactation is usually low and is also highly variable among piglets in a litter and between litters (Barnett *et al*., 1989; Pajor *et al*., 1991; Kuller *et al*., 2007). Stable diversity indices and a high similarity index between DGGE profiles of samples taken from weaned piglets 35 and 42 days of age demonstrated that a stable commensal microbiota in the gut of healthy piglets is building over time after weaning.

In this study, several microbial groups that have previously been described by using cultivation methods as normal inhabitants of the intestine of healthy adult pigs, *Eubacterium*, *Bacteroides*, and *Prevotella,* were not identified (Stewart *et al.,* 1997). It is possible that *Eubacterium*, *Bacteroides*, and *Prevotella* species only represent a small proportion of the microbiota in feces of nursing piglets studied here and could not be detected with universal primers. Similarly, *Eubacterium* and *Ruminococcus* were not identified in fecal samples of weaned piglets that were fed a diet with FOS and SBP for 13 days (Konstantinov *et al.*, 2003). This reinforces the notion that diets can significantly affect the composition of commensal microbiota of the host pig. On the other hand, it is possible that some bacterial groups are preferentially amplified with universal bacterial primers and vice versa. In addition, it can not be excluded that the size of the clone library analyzed in this study was not sufficient to detect less abundant populations. Similarly, it has been reported that DGGE analysis with universal bacterial primers is sensitive enough to visualize populations that constitute as little as 1% of the total bacterial community (Zoetendal *et al*., 1998 and 2001). To unambiguously confirm the absence of these organisms, or rather detect their presence, application of other, complementary molecular approaches would be required, such as PCR-amplification or in situ hybridization with group-specific primers or probes.

Our investigations were intended to study microbiota in creep fed piglets before and after weaning. We aimed at the fecal microbiota of these piglets which suffered from diarrhea directly after birth. We were able to show that early creep-feeding stabilizes

the microbiota of piglets around the weaning period. On the other hand, early creep-feeding can increase the instability of microbiota of suckling piglets. Therefore, the development of new strategies for helping suckling piglets towards a rapid development of a balanced and healthy commensal gut microbiota is required to prevent the diarrhea of suckling piglets, and needs to be addressed in future research.

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CHAPTER 4

Cultivation-independent Analysis of the Development of the *Lactobacillus* spp. Community in the Intestinal Tract of Newborn Piglets

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Abstract

Molecular diversity and development of the *Lactobacillus* community in the intestinal tract, as influenced by age, diet and intestinal compartment, were studied in one litter of 12 conventionally raised piglets. Piglets were euthanized at 1, 2, 3 (weaning) and 4 weeks of age (3 animals at each day). Digesta and tissue samples from stomach, duodenum, jejunum, ileum, caecum, colon and rectum were collected and analysed by using 16S ribosomal RNA-based methods. DGGE profiles revealed that the *Lactobacillus* communities throughout the GI tract from duodenum to rectum showed good stability at same age. This indicates that fecal *Lactobacillus* communities can effectively represent the intestinal community. Two dominant bands were found in tissue samples of the small intestine, suggesting that the lactobacilli can adhere to the small intestinal wall. The *Lactobacillus* communities in different GI tract compartments developed over time. A successional change of *Lactobacillus* communities was observed from birth, through creep feeding to one week after weaning, showing a trend from simple to complex and back to simple. Furthermore, a clone library of *Lactobacillus* spp. 16S rRNA gene sequences was generated from jejunal and colonic chymes. Six dominant DGGE bands generated from jejunal chymes were matched with sequences that show 94-98% similarity to the bands derived from *L. reuteri, L. delbrueckii,* and *L. crispatus*. Seven dominant DGGE bands generated from colon chymes were matched with sequences that show 88-99% similarity to those derived from *L. reuteri, L. delbrueckii, L. amylovorus/L. sobrius,* and *L. acidophilus*. Amplicons related to *L. reuteri* were found in all DGGE fingerprints from jejunal digesta of age of week 1, 3 and 4. Amplicons related to *L. amylovorus/L. sobrius* were present in all DGGE fingerprints from colonic digesta of age of week 1, 3 and 4. Amplicons related to *L. delbrueckii* were found before weaning, *L. crispatus* after creep feeding before weaning, and *L. acidophilus* after weaning. This indicates that *L. reuteri* and *L. amylovorus/L. sobrius* probably belong to the permanent composition, while *L. delbruckii, L. acidophilus* and *L. crispatus* probably belong to the temporal groups of *Lactobacillus* communities in the GI tract of piglets.

Keywords: Molecular diversity; 16S rRNA gene; Denaturing gradient gel electrophoresis

(DGGE); *Lactobacillus* community; piglets

1. Introduction

Mammals are born without microorganisms (Mackie *et al*., 1999). Starting at birth, the microbiota must develop from a simple unstable community into a complex community. This process can be influenced by diet, environmental factors, and by the host itself (Konstantinov *et al*., 2004a). All the members of the gastrointestinal microbiota are needed for the gut to develop its specific intestinal functions (Hooper and Gordon, 2001). Lactobacilli are considered beneficial because they are important for maintaining a healthy, balanced microbial community in GI-tract. It is considered that they directly affect the host's health. It has been postulated that *Lactobacillus* spp. has several health-promoting effects. These include 1) immuno-stimulation by association with intestinal mucosa, 2) alleviation of food intolerance and allergy by production of hydrolytic enzymes, 3) prevention of diarrhea and intestinal infections by production of Lactic acid, acetic acid and antimicrobials (Servin, 2004; Bengmark *et al*., 2000; Ouwehand *et al*., 1999; Salminen *et al*., 1998). Consequently, *Lactobacillus* spp. have been the most frequently used probiotic strains for disease treatment and prevention as well as health restoration and maintenance in humans.

Nursing is a major critical period of pig rearing. Creep-feeding in nursing period is commonly used in Chinese swine production (Huang 2003). Over the last decades, the preventive use of antibiotics and metals (copper and zinc) in creep-feeds are used in nursing management in China. These practices, however, have increased bacterial resistance to antibiotics and environmental problems. It can be expected that in future also China will follow the European Union to consider a full ban on the use of in-feed antibiotics and a drastic reduction in the levels of usage of copper and zinc in the future. The concept of probiotic, which is defined as a live microbial feed supplement that is beneficial to health (Salminen and others 1998) and is considered as an alternative to antibiotics, attracts increasing interests of animal nutritionists and livestock producers also in China. In swine the most frequently used strains are *Enterococcus faecium* (natural habitant: digestive tract) because of its readily cultivatable ability and *Bacillus spp.* (natural habitat: soil) because of its high survival during feed processing. Recently, the use

of species and strains of lactobacilli, such as *L. reuteri* (Simpson *et al*., 2000) and *L. plantarum* (van Winsen *et al*., 2001; Demeckova *et al*., 2002), is increasing. This is due to the possible benefits of lactobacilli for porcine gut function and health. With probiotic treatment the incidences of diarrhea were reduced significantly in the most feeding trials after weaning. Trials in literature which are aimed on improvements of growth performance showed significant results in some feeding trials only. There is a high variation between individual animals in the response to probiotics (Simon, 2005).

Although considerable efforts have been made with regard to the efficiency and the mode of action of probiotics, our understanding is still far from complete. Whereas it is generally assumed that there is more than one mode of action, one of the major principles is the modification of intestinal bacterial populations by probiotics. Probiotics inhibit the growth of pathogen bacteria, reduce the translocation of bacteria (Meier and Lochs, 2007). Their effectiveness depends on the microbial status of a flock of animals and of the individual animal. Abbott (2004) characterized this situation in Nature as follow “even when probiotics seem to work, we know too little about the normal gut ecosystem to understand why.” *Lactobacillus* spp. should be one of microbial groups of interest, as it has possible benefits for gut function and health of the host.

Our current understanding of the porcine GI tract microbiota largely depends on cultivation (Moore *et al*., 1987; Robinson *et al*., 1988; Salanitro *et al*., 1977). Culture-based methods only provide information on bacteria that are readily cultivated, and can give a biased view of microbial diversity. *Lactobacillus* was found to be a predominant genus by plate count, but the analysis of intestinal microbial diversity by oligonucleotide probe hybridization revealed that they constitute less than 1% of the total bacteria community (Sghir *et al*., 2000). DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments, however, provide a powerful tool to visualize the gut microbial community. It has been reported that DGGE is sensitive enough to visualize populations that constitute at least 1% of the total bacterial community. Therefore, using universal bacterial primers, it is nearly impossible to monitor this group. Hellig *et al*. (2002) consequently developed a *Lactobacillus*-specific PCR in combination with DGGE to monitor the molecular diversity of this group. The authors concluded that this strategy is widely applicable and allows for the characterization of bacteria present in low numbers

in the human gut. Konstantinov *et al*. (2004b) then used this approach to investigate the effect of a fermentable carbohydrates-containing diet on the ileum *Lactobacillus*-like community of weaned piglets.

The primary aim of the present investigation was to describe the intestinal *Lactobacillus* microbiota composition in piglets. It is also aimed to study their succession by using *Lactobacillus* group-specific PCR primers with DGGE when piglets followed the actual Chinese feeding strategy during the neonatal and weaning period.

2. Materials and methods

2.1 Experimental approach

To describe the development and diversity of the *Lactobacillus* community in the piglet GI tract, total DNA was isolated from digesta and tissue samples from different gastrointestinal compartments, and used as a template for PCR amplification. Nested PCR amplicons of the V2-V3 regions of *Lactobacillus* 16S rRNA genes were analyzed using DGGE (Muyzer *et al*., 1993). Clone libraries of V1-V3 regions of *Lactobacillus* 16S rRNA gene amplicons were generated from jejunum and colon digesta samples. Cloned amplicons that showed the same mobility on DGGE gel as specific bands of intestinal samples were further characterized by nucleotide sequence analysis. All the procedures involving animals were conducted in accordance with the Chinese law on experimental animals.

2.2 Animals, diets and sampling

The trial was done with 12 healthy crossbred piglets (Yorkshire × Erhualian) of one litter. It started at birth and lasted to 4 weeks of age (1 week after weaning). Creep feeding was done with Agribrands Purina Nanjing Feedmill Co., Ltd feed. The composition of creep feed was as follows: 70.5 percent of maize, 20 percent of soybean meal, 5.5 percent of fishmeal and 4 percent of premix. The creep feeding was started at 1 week of age. Piglets were weaned at 3 weeks of age. Three piglets were euthanized at 1, 2, 3 (pre-weaning) and 4 weeks of age, respectively. For biomolecular analyses, digesta and tissue samples were collected immediately after sacrifice from duodenum, jejunum, ileum, caecum, colon and rectum of each piglet. Samples were kept at -20°C until further processing.

2.3 DNA isolation

The DNA of digesta samples was extracted from 300-mg of digesta using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad. CA). The tissue samples were ground, and DNA isolation was performed by a bead-beating method as previously described (Zoetendal *et al*., 1998). Electrophoresis in agarose gels 1.2% (wt/vol) containing ethidium bromide was used to check the amount of DNA visually.

2.4 PCR amplification

To investigate the *Lactobacillus*-like intestine bacterial community by DGGE, PCR with primers Bact0011f (5'-AGA GTT TGA TCC TGG CTC AG-3') and Lab0677r (5'-CAC CGC TAC ACA TGG AG-3'), and the nested PCR with primers Lab159f (5'-GGA AAC AGG TGC TAA TAC CG-3') and Univ0515rGC (5'-ATC GTA TTA CCG CGG CTG CTG GCA -3') by using 100-fold diluted products from previous amplification with Bact0011f and Lab0677r as template, were performed according to the protocols described previously (Heilig *et al*., 2002).

2.5 DGGE analysis of PCR amplicons

PCR products generated with Lab159f and Univ0515rGC were separated by DGGE according to the specification of Muyzer *et al.* (1993), using the Dcode system (Bio-Rad Laboratories, Hercules, CA). The gels were poured from the bottom by using a gradient maker and a pump (Econopump, Bio-Rad) set at a speed of 4.5ml/min. A gradient of 30% to 50% was used for the separation of the generated amplicons. Electrophoresis was performed for 16h at 85 V in a 0.5× TAE buffer at a constant temperature of 60°C. Gels were then stained with $AgNO_3$.

All DGGE gels were scanned at 400 dpi. The Dice similarity coefficient and the Shannon index of diversity (Shannon, 1963) of the DGGE profiles were calculated based on image analyses of gels by using the Molecular Analyst software (version 1.12, BioRad).

2.6 Cloning and Sequencing of the *Lactobacillus*-specific PCR-amplified products

PCR amplicons with Bact0011f and Lab0677r were purified using the Qiaquick PCR purification kit (Westburg, Leusden, the Netherlands) according to the manufacturer's instructions. Purified PCR products were further cloned in *E. coli* JM109 using the

Promega pGEM-T vector system (Promega, Madison, WI). To confirm the size of inserts, PCR with pGEM-T specific primers T7 (5'-TAA TAC FAC TCA CTA TAG G-3') and Sp6 (5'-GAT TTA GGT GAC ACT ATA G-3') was performed on lysates of ampicillin-resistant transformants. Amplicons of the correct size were subjected to restriction fragment length polymorphism analysis by using restriction enzyme MspI. Plasmids containing a unique insert of the appropriate size were compared with the original samples on DGGE gels. Plasmids corresponding to dominant bands of the DGGE profile were purified by the QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and were subjected to DNA sequence analysis.

Purified pGEM plasmid was used for sequence analysis of the cloned 16S rRNA gene fragments. Sequencing reactions were performed with the Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, United Kingdom) according to the manufacturer's specifications by using the IRD-800 5'-primer end-labeled primer T7. Sequences were automatically analyzed on a LI-COR DNA sequencer 4000L (Lincoln, Nebr.) and corrected manually.

2.7 Sequencing of DGGE bands

Representative bands were excised from DGGE gels using a QIAEXII Gel extraction kit (Westburg) according to the instructions in the manual. After re-amplification using the original Lab159f and Univ0515rGC primer set, the PCR products were subjected to sequence analysis.

2.8 Nucleotide sequence accession numbers

14 partial 16S rRNA gene sequences determined in this study were deposited at the GenBank database under accession numbers AY662519 - AY662532.

2.9 Statistical analysis

The data of Shannon diversity index and the Dice similarity coefficient were expressed as the mean±SE. The Student's *t*-test was used to determine the statistical significance of the differences between mean values of Shannon indices.

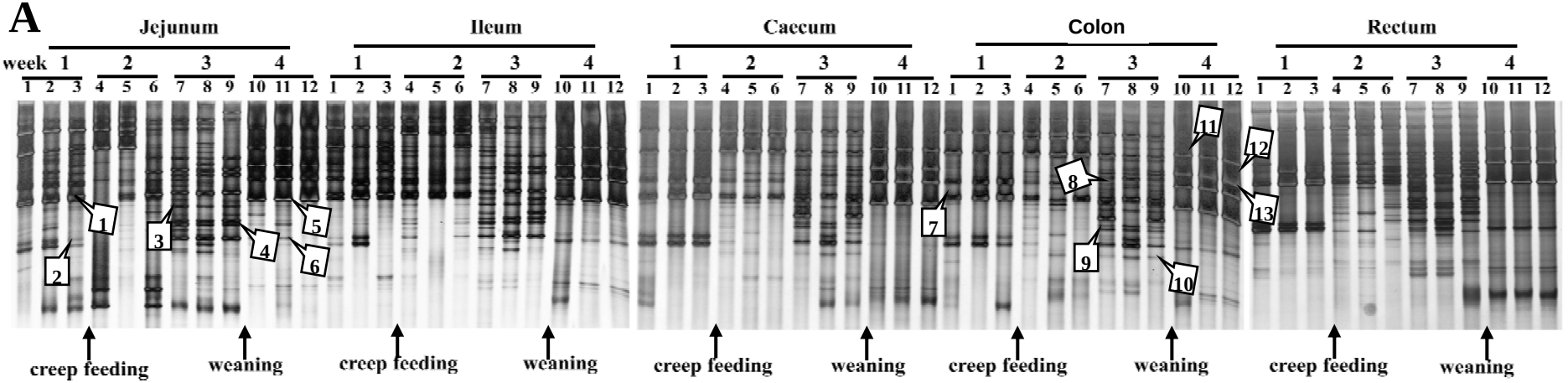
3. Results

3.1. DGGE analysis for description of intestinal *Lactobacillus* composition of piglets and identification of cloned 16S rRNA gene sequences in DGGE profiles.

DGGE profiles were generated from intestinal digesta samples taken from neonatal piglets during the first 4 weeks of life (Figure 4.1A). The bands in DGGE profile represent the majority of the dominant *Lactobacillus* populations in the community, and their appearance and disappearance reflect approximate changes in the *Lactobacillus* community composition. The intensity of a band provides a rough estimate of the proportion of the corresponding population in a sample. The *Lactobacillus* communities in each compartment changed from simple to complex and returned to simple during this four weeks period. The most complex banding pattern appeared at week 3. There were several new bands (or new lactobacilli) only observed at this time, and disappeared again at week 4.

In order to identify the species corresponding to certain bands observed in the DGGE gels, 16S rRNA gene fragment clone libraries were prepared from a number of digesta samples obtained at various ages. The mobility of the cloned fragments was compared to the mobility of fragments amplified from the original samples by DGGE, and the DNA sequences of clones that corresponded to dominant bands were determined. Clone ID, accession number and their closest relative are given in Figure 4.1B. Six dominant DGGE bands generated from jejunal digesta were matched with sequences that show 94-98% similarity to those derived from *L. reuteri, L. delbrueckii,* and *L. crispatus*. Seven dominant DGGE bands generated from colon digesta were matched with sequences that show 88-99% similarity to those derived from *L. reuteri, L. delbrueckii, L. amylovorus,* and *L. acidophilus*. Amplicons related to *L. reuteri* (clone ID: YRJ37) were found in all DGGE fingerprints from jejunal digesta of age of week 1, 3 and 4. Amplicons related to *L. amylovorus* (clone ID: YRC86) were presented in all DGGE fingerprints from colon digesta of age of week 1, 3 and 4. Amplicons related to *L. delbrueckii* were found before weaning, *L. crispatus* in the stage of creep feeding, and *L. acidophilus* after weaning.

A



B

N	Closest Relative	Identity %	Clone ID	Accession	N	Closest Relative	Identity %	Clone ID	Accession
1	*Lactobacillus delbrueckii subsp. lactis*	98	YRJ9	AY662527	7	*Lactobacillus delbrueckii subsp. lactis*	96	YRC4	AY662519
2	*Lactobacillus sp. oral clone* CX036	95	YRJ28	AY662528	8	*Lactobacillus delbrueckii subsp.bulgaricus*	97	YRC89	AY662523
3	*Lactobacillus crispatus* strain TSK V38-1	99	YRJ39	AY662530	9	*Lactobacillus delbrueckii*	92	YRC69	AY662521
4	*Lactobacillus delbrueckii*	94	YRJ58	AY662531	10	*Lactobacillus amylovorus*	98	YRC86	AY662522
5	*Lactobacillus reuteri*(DSM20016T)	97	YRJ100	AY662532	11	*Lactobacillus acidophilus johnsonii*	95	YRC100	AY662525
6	*Lactobacillus reuteri*(DSM20016T)	98	YRJ37	AY662529	12	*Lactobacillus sp.* G35	88	YRC95	AY662524
					13	*Lactobacillus reuteri*(DSM20016T)	97	YRC37	AY662520

Figure 4.1 (A) Succession of the Lactobacillus community of digesta during the first month in the piglet intestinal tract. Changes of feeding strategy are indicated by arrows. The bands identified from the 16S rDNA clone libraries are numbered and are indicated by arrowheads. **(B)** Closest relative as determined by comparative sequence analysis, identity with this relative, clone ID, and accession number for each band identified in panel A.

3.2. Effects of age and feeding strategy on the succession of the piglets' *Lactobacillus* community.

During this four-week period, there was a shift in feeding strategy. This involved sow's milk only in week 1, sow's milk with creep-fed in week 2 and 3, weaning at day 21 and solid diet after weaning in week 4. This is a general feeding strategy used in China. At sacrifice we observed that the duodenum, jejunum, ileum, caecum and colon had only a small amount of digesta at week 1. Afterwards the GI tract continuously enlarged in each intestinal compartment for both the length and the diameter. The amount of digesta increased definitely with age except in the foregut of 2 week old piglets.

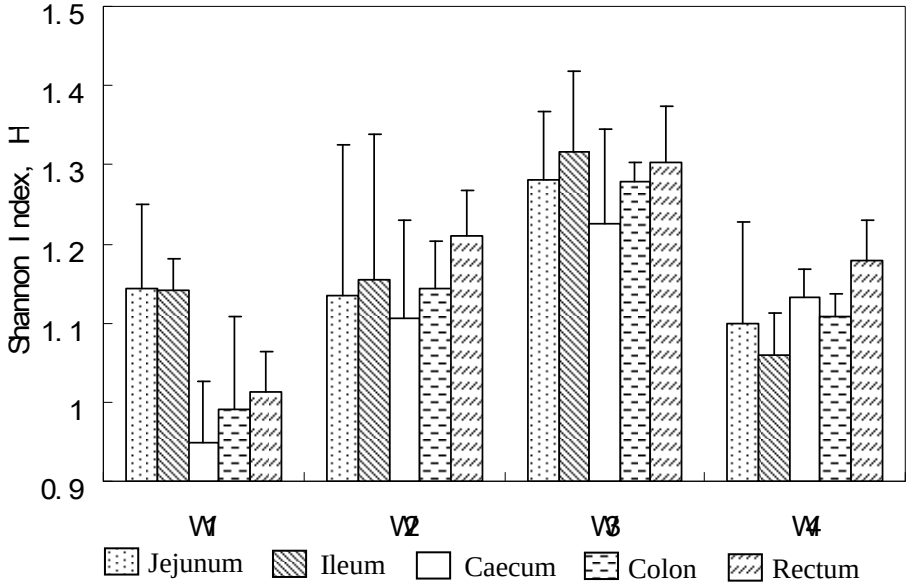


Figure 4.2 Diversity index of DGGE banding pattern created from digesta sample of different intestinal compartments in time week 1, 2, 3, 4.

To objectively describe the succession of the piglets' *Lactobacillus* community in different compartments along the entire intestinal tract, we calculated Shannon indices (Figure 4.2) of DGGE profiles presented in Figure 4.1. This diversity index analysis showed differences in the banding patterns and in relative band intensities between samples. There is a low diversity in microbiota during the first two weeks, a high diversity in the third week, and low diversity in the fourth week again. In week 1, the diversity indices from foregut DGGE profiles (1.14±0.11 of jejunal digesta and 1.14±0.04 of ileal digesta) were higher than those from hindgut DGGE profiles (0.95±0.08 of caecum digesta, 0.99±0.12

of colon digesta and 1.01±0.05 of feces). The differences between ileal and each compartment of the large intestine are significant. After creep feeding, the situation reversed quickly. High diversity values were calculated from hindgut DGGE profiles in week 4 (1.13±0.04 of caecum digesta, 1.11±0.03 of colon digesta and 1.18±0.05 of feces comparing with 1.09±0.13 of jejunal digest and 1.06±0.05 of ileal digesta). Significant differences were observed within ileum, colon and rectum.

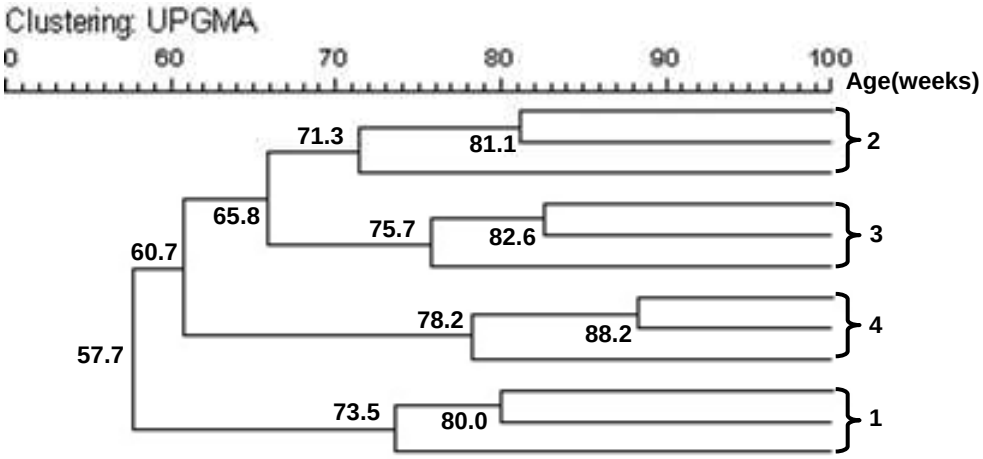


Figure 4.3 Similarity dendrogram of the DGGE profiles from fecal samples of piglets. 12 piglets of one litter slaughtered at the age of 1, 2, 3, and 4 weeks, three piglets euthanized at one time point. The DGGE profiles of these fecal samples were presented in Fig 4.1 (labeled as Rectum).

To assess the effect of feeding strategy on this succession of piglets' *Lactobacillus* community, the DGGE profiles from feces as presented in Figure 4.1 were submitted to a numerical analysis based on the Dice similarity coefficient followed by cluster analysis. The similarity was visualized using the UPGMA algorithm (Figure 4.3). Cluster analysis revealed 9 fecal samples of piglets after creep-feeding formed a coherent cluster with similarity above 60.7%, while 3 fecal samples of piglets before creep-feeding formed another cluster with similarity above 73.5%. A low similarity of 57.7% was observed between these two groups, indicating that creep-feed changes the composition of *Lactobacillus* community of piglets. Within the cluster after creep-feeding, 6 pre-weaning piglets formed a subgroup with similarity above 65.8%, 3 weaned piglets formed another subgroup with similarity above 78.2%, indicating that weaning could also change the composition of *Lactobacillus* community of piglets. Theses results confirmed the visually

observed succession of piglets' *Lactobacillus* community in their DGGE fingerprints (Figure 4.1) and suggested that the feeding strategy (one week age creep feeding, three weeks age weaning) was the main source of this succession.

3.3. Effects of sampling site within the gut on *Lactobacillus*-specific DGGE profiles, and adherence of lactobacilli to the small intestinal mucosa.

To objectively evaluate the effect of sampling site on *Lactobacillus* composition, we calculated the similarity indices of the DGGE profiles from jejunum, ileum, caecum, colon and rectum digesta samples (Figure 4.4). At 1 and 4 weeks of age, high similarity indices (over 90%) were found between adjacent compartments, and between foregut and hindgut. This indicated that fecal *Lactobacillus* composition can represent those within the intestine properly. At 2 weeks of age, 71.8% of similarity was observed between jejunum and ileum, 73.4% of similarity between jejunum and colon, 83.3% of similarity between ileum and colon, and 89.9% of similarity between colon and rectum. At 3 weeks of age, 83% of similarity was observed between jejunum and colon, 88.8% of similarity between jejunum and colon, 97% of similarity between ileum and colon, and 88.8% of similarity between colon and rectum. Thus, creep-feeding and weaning mildly decreased the similarity.

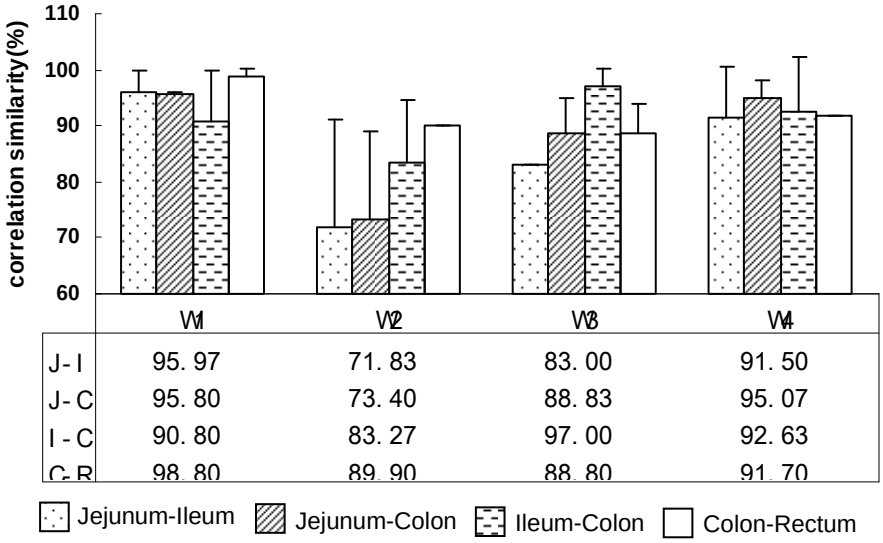


Figure 4.4 Comparison of the DGGE profiles of the digesta samples from different intestinal compartments.

In addition, we also calculated the similarity for DGGE profiles obtained from samples of gastrointestinal mucosa and corresponding digesta of piglets at 1 week of age (Figure 4.5). Except for the small intestine (duodenum, jejunum and ileum), high similarity indices (over 97%) were found between mucosa and digesta in the other gut compartments. Two dominant bands were found in profiles obtained from mucosa samples of duodenum and jejunum that were not observed in the corresponding digesta-derived profiles, suggesting that these *Lactobacillus* populations can adhere to the small intestinal mucosa (Figure 4.6A). To confirm this observation, piglets from another litter, which followed same feeding strategy, were euthanized at 7, 14, 21, 24 and 35 days of age in a separate trial (detail animal experimental design see in chapter 7). Samples from mucosa and digesta of jejunum and ileum were subjected to *Lactobacillus*-specific PCR/DGGE analysis (Figure 4.6B). Profiles also revealed the presence of two mucosa-specific bands. Bands were excised from the gel, re-amplified and subjected to sequence analysis. One band showed 88% of similarity to *Lactobacillus casei*, the other band was failed to have its sequence, indicating that they are uncultured and unidentified lactobacilli.

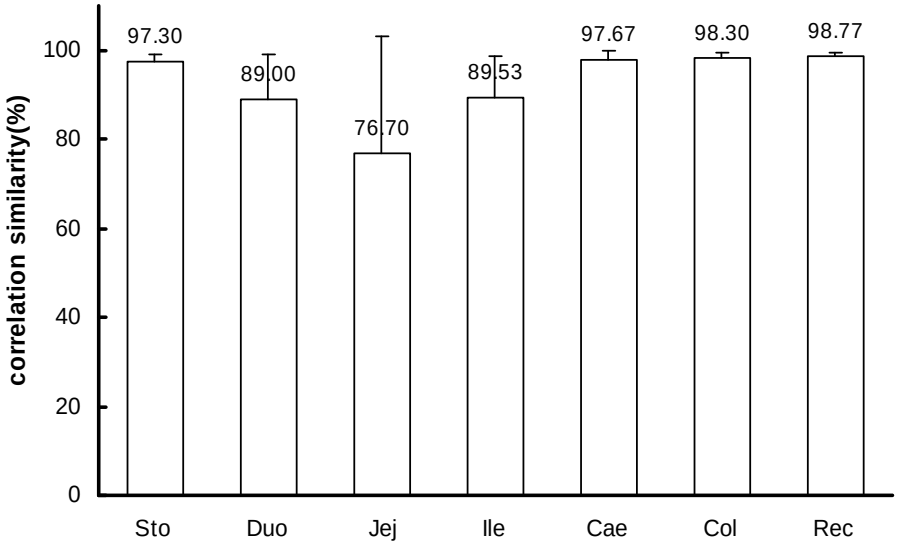


Fig 4.5 Comparison of the DGGE profiles of the samples from gastrointestinal mucosa and lumenal digesta, piglets slaughtered at 1 week of age.

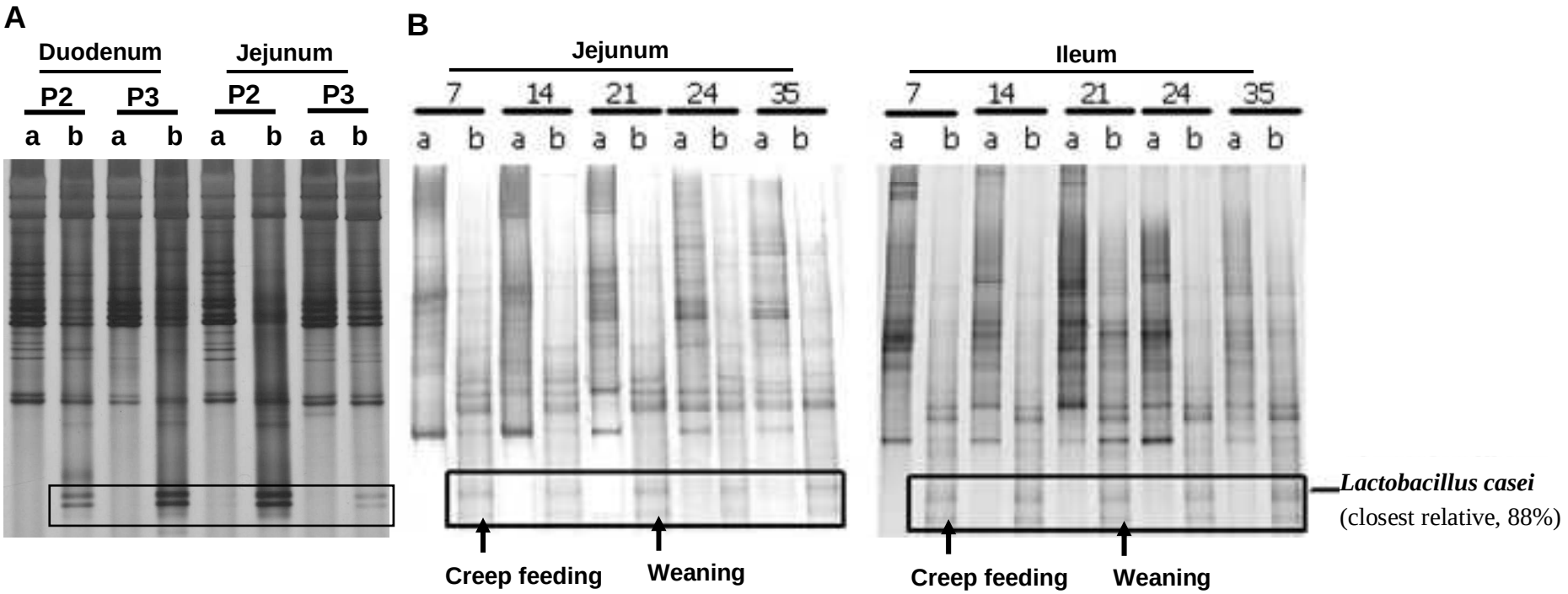


Figure 4.6 (A) Comparison of the Lactobacillus community derived from digesta and mucosa of duodenum and jejunum, piglets slaughtered at day 7. P2 and P3 were the number of piglets which were same with those in Figure 4.1 (A). **(B)** Succession of the Lactobacillus community derived from digesta and mucosa of jejunum and ileum, piglets slaughtered at day 7, 14, 21, 24 and 35. Creepfeeding was from 7 days of age, and weaning was from 21 days of age. These samples were from piglets used in chapter 7.

4. Discussion and Conclusion

Weaning is a major critical period of pig rearing because of increased susceptibility to gut disorders, infections and diarrhea. It has been reported that intake of a sufficient amount of creep feed during suckling creates a more gradual transition at weaning and can reduce the occurrence of postweaning disorders. However, creep feed consumption during lactation is usually low and is also highly variable among piglets within and between litters (Barnett *et al*., 1989; Pajor *et al*., 1991; Kuller *et al*., 2007). Research so far mostly focused on growth performance, while Mathew *et al*. (1994) reported that creep feeding did not affect lactobacilli or *E. coli* concentrations after weaning. Shim *et al*. (2005) observed the effects of antibiotic-free creep feed with oligofructose (OF), probiotics or synbiotics on the composition of intestinal microbiota of suckling piglets, using culture-based methods. This showed that piglets fed the OF, probiotics or synbiotics diet had a significantly decreased number of total coliform bacteria in the colon. Feeding OF, probiotics or synbiotics significantly increased the population of bifidobacteria in the ileum compared to control animals. In the colon, the probiotics and synbiotics diet significantly increased the number of bifidobacteria compared with the control diet. To our knowledge, the present study is the first application of a *Lactobacillus*-specific PCR in combination with DGGE to monitor the succession of the *Lactobacillus*-like community of nursing piglets and the effect of creep-feed on it.

In our study, DGGE profiles generated from digesta samples of weeks 1 and 4 shared several pre-dominant bands, and had an overall similarity of 57.7 %. This indicates that the first colonizers, which the piglets most likely received from their mother, were able to become the general members of the fairly mature and stable commensal *Lactobacillus*-like community after weaning. It is known that 14 day old piglets consume 0-25 g of creep-feed in addition to 25 g of sow’s faeces daily (Ewing and Cole, 1994). Hence, it can be assumed that sow’s feces most probably is one of the main sources for inoculation, and that diets and sow’s feces are important in the establishment of the gut microbiota. This may explain why a highly diverse *Lactobacillus*-like community was observed in each intestinal site of piglets at 3 weeks of age, and the highest diversity indices of each intestinal site were calculated at this age.

DGGE analysis revealed distinct total bacterial communities in different gut compartments of one 5-month-old pig (Simpson *et al*, 1999). In our study, a high similarity (with over 90% of similarity indices showed in Fig 4) was found in all intestinal sites of piglets at 1 and 4 weeks of age. A moderate decrease was observed at 2 and 3 weeks of age when piglets were exposed to creep-feeding and weaning. Only moderate differences were observed between the lactobacilli-like communities present in the fore- and the hindgut. The characteristics of the gut of piglets during the nursing period, and the property of *Lactobacillus* spp. may be the main reason for this similarity. For nursing piglets, there are no big differences with respect to the chemical conditions of the foregut and hindgut because of the fairly high pH value in the foregut and less fermentation in the hindgut. Volatile fatty acids are toxic to gram-negative bacteria (Ewing and Cole, 1994). Lactobacilli are gram-positive bacteria, and have an increased tolerance to low pH and bile, although there is a wide variation in bile sensitivity (Mishra, 2005). Our results showed that the *Lactobacillus*-like communities from the duodenum to the rectum of piglets at the same sampling time were fairly similar, indicating that fecal *Lactobacillus*-like communities could effectively represent those that are present at more upstream intestinal locations. Thus, using a *Lactobacillus*-specific PCR in combination with DGGE to monitor fecal microbiota is an applicable and effective method for the description of inner-intestinal changes without slaughtering piglets.

Culture-based studies showed that bacteria remain in the gut in two ways: by attachment to the epithelial cells lining the intestine, and by growing at a rate faster than the rate at which they are being removed by peristalsis (Ewing and Cole, 1994). Through the ability to adhere to and to colonize intestinal tissues, probiotic microorganisms can prevent pathogen access by steric interactions or specific blockage of cell receptors, and stimulate their removal from the infected intestinal tract (Lee *et al*. 2000). This phenomenon has been known as “bacterial interference”. In this study, we observed two dominant bands specifically in small intestinal mucosal samples, suggesting firm adherence of the corresponding populations to the mucosa. No firm adhesion, however, was found in case of the large intestine, as evidenced by the absence of the specific bands in DGGE profiles derived from colonic mucosa samples. One of the specific bands showed 88% of sequence similarity to the 16S rRNA gene of *Lactobacillus casei*. *L. casei* has been used

successfully as a probiotic in traditional milk products, as well as in cheeses and a number of commercial fermented food products, but it has not been reported as a dominant resident of the porcine gut microbiota. The identity of the mucosa-associated populations observed in this study, as well as their ecophysiology and the mechanism of their adhesion will be addressed in future research efforts.

Konstantinov *et al*. (2006a) generated clone libraries from ileal lumen samples of two newborn (2 days old), two weaned and two unweaned piglets (23 days old). All piglets had no creep feed during the nursing period. This analysis revealed that in the samples from neonatal and unweaned piglets, approximately 30% of all clones matched (**>**98%) with the 16S rRNA of *L. reuteri, L. amylovorus*-like, and *L. acidophilus, L.amylovorus*-like 16S rRNA gene amplicons, however, were not detected in the weaned group samples. Comparing to this research, we detected *L. reuteri, L. delbrueckii,* and *L. crispatus* from jejunal digesta, *L. reuteri, L. delbrueckii, L. amylovorus,* and *L. acidophilus* from colon digesta. Different diet composition and feeding strategy might be a reason for the observed discrepancies. In China corn replaces wheat and barley grains in swine diets for energy. Creep feeding from 1 week of age and weaning at 3 weeks of age are the main elements of the currently applied general feeding strategy in China. Creep feeding provides the piglets with the opportunity to intake solid diets during the same time of suckling. In our study, bands corresponding to *L. reuteri* were found in all DGGE fingerprints from jejunal digesta at 1, 3 and 4 weeks of age. *L. amylovorus* was present in all DGGE fingerprints from colon digesta at 1, 3 and 4 weeks of age. *L. delbrueckii* was found before weaning, *L. crispatus* after creep feeding before weaning, and *L. acidophilus* after weaning. This indicated that *L. reuteri* and *L. amylovorus* are fairly stable members of the porcine intestinal lactobacilli-like community, while other populations might be more transient members. In this respect, it is noteworthy that we and others were able to obtain a pure culture isolate of *L. amylovorus* (the strain named as *Lactobacillus sobrius* S1), allowing the study of eco-physiology as well as probiotic properties of this important early colonizer of the pig intestine in further studies (Chapter 7, Konstantinov, 2005; Konstantinov *et al.*, 2006b).

In conclusion, *Lactobacillus* communities follow a successional change associated with piglet growth and diet shifting. Creep feeding stabilizes the *Lactobacillus* community of

weaning piglets. Within the *Lactobacillus* community, some members like *L.reuteri* and *L.amylovorus/L.sobrius* might be permanent colonizers, while *L.delbrueckii*, *L.acidophilus* and *L.crispatus* might be transient members of the *Lactobacillus* communities in the piglet´s GI tract.

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CHAPTER 5

Daidzein *in vitro* Increased Lactic Acid Production and the Density of Lactobacilli but did not Change Composition of *Lactobacillus* Community in Piglet Digesta

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Abstract

Four separate *in vitro* fermentation batches paralleled with slaughtering time were conducted to investigate the impact of the phytoestrogen daidzein on compositional and functional aspects of the porcine ileal and colonic *Lactobacillus* community during the nursing period. One litter of 12 conventionally raised piglets was used and euthanized at 1, 2, 3 (weaning) and 4 weeks of age (3 animals each). Ileal and colonic chymes were used as inocula in *in vitro* treatments, respectively: Van Soest medium with (a) 0.5g of glucose and 5mg of daidzein, (b) 0.5g of glucose, and (c) Van Soest medium only. After 48 h of fermentation, volatile fatty acid (VFA) profile, lactic acid concentration and MRS plate count of lactic acid bacteria (LAB) were analyzed from the culture liquid. Culture DNA was analyzed by *Lactobacillus* specific 16S ribosomal RNA gene-targeted PCR in combination with denaturing gradient gel electrophoresis (DGGE). Dilution PCR with primers Lab0677r and Bact0011f was used to assess relative abundance of lactobacilli. Within each intestinal compartment, i.e. ileum and colon, respectively, DGGE patterns became more complex as piglets grew from 1 to 3 weeks (before weaning) and then less diverse in week 4. On week 1, the ileal microbiota produced more gas, more total volatile fatty acid (TVFA) and more lactic acid than the colon microbiota. On week 4, more gas and TVFA were produced in the colonic fermentation. Daidzein did not affect DGGE patterns of *Lactobacillus* community. Dilution PCR, however, indicated that daidzein increased the relative abundance of *Lactobacillus* irrespective of intestinal compartment and piglet age. This was confirmed by MRS plate counts and lactic acid production, and the results suggest that daidzein may have the potential for use as a prebiotic substance in animal feed.

Keywords: Denaturing gradient gel electrophoresis (DGGE), *Lactobacillus* community, Daidzein, Dilution PCR, Plate-counting

1. Introduction

As piglets grow from birth to adult, their gut microbial community develops from simple to diverse and becomes established (Konstantinov *et al*., 2004). Thus during growth the piglet gut is suitable for manipulation. On one hand, creep-feed can influence gut microbiota during suckling (Mathew *et al*., 1994). Also weaning stresses can impair microbiota and increase susceptibility to gut disorders like infections and diarrhea (Lalles *et al*, 2004). On the other hand, some currently used alternatives to in-feed antibiotics, such as probiotics and prebiotics, can modulate this un-established microbiota to prevent gut disorders and infections (Roselli *et al*., 2005).

Daidzein, a phytoestrogenic compound, is produced in plants of the legume family, especially in soybean. It has been concluded from a recent review that daidzein can promote male animal growth, induce female mammary development and lactation, and improve laying performance of laying birds (Han *et al*., 2006). According to the metabolic pathway of daidzein proposed in humans, based on the isoflavone metabolites found in human urine, daidzein is converted via dihydrodaidzein to both equol and O-desmethylangolensin (O-DMA) by intestinal microorganisms (Setchell *et al*., 1988; Joannou *et al*., 1995). The metabolic fate of daidzein, however, differs among individuals in humans and monkeys due to individual differences in gut microbiota composition (Rafii *et al*., 2003, 2004; Atkinson *et al*., 2004). This can be seen as a reason for individual variation in the effects of daidzein on humans. Similar inter-individual differences have been observed in animal trials, and differences in basic levels of metabolic hormones of individual animals have been proposed as the underlying reason (Han *et al*., 2006). It is not clear whether the variation in intestinal microbiota is involved in this individual variation. Moreover, the direct effect of daidzein on the pig intestinal microbiota is largely unknown.

De Boever *et al*. (2000) studied the effect of a soygerm powder rich in β-glycosidic isoflavones on the fermentation pattern of the colon microbiota of healthy humans by using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The supplement of 2.5 g/d of soygerm powder which provided 62.5 mg/d of isoflavone promoted an overall increase of populations of *Enterobacteriaceae*, coliforms, *Lactobacillus* spp., *Staphylococcus* spp. and *Clostridium* spp., with a significant increase

of the *Lactobacillus* spp. population. The short-chain fatty acid (SCFA) concentration increased 30% during the supplementation period, which was due mainly to a significant increase of acetic and propionic acids. This study indicated that the consumption of soygerm powder may beneficially influence the gut microbiota, however, this beneficial effect could not be completely attributed to isoflavones due to its low content in soygerm powder.

The aim of this study, therefore, is to evaluate by means of an in vitro system the impact of dadzein on compositional and functional aspects of the ileal and colonic *Lactobacillus* community of piglets of different ages.

2 Methods and materials

2.1 Experimental design

This study was conducted as four separate *in vitro* fermentation batches. Each batch is a euthanization age of piglets. Ileal and colonic chyme of 12 piglets euthanized, with 3 piglets at 1, 2, 3 and 4 weeks of age, respectively, were used as inocula. Three treatments for each inoculum (ileum digesta and rectum faeces) within each fermentation batch, (a) Van Soest medium + 0.5g of glucose + 5 mg of daidzein, (b) medium + 0.5g of glucose and (c) medium only (blank), were designed in this study. Daidzein was provided by the Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture of the People's Republic of China. Each inoculum has three replicate bottles. Each bottle contained 90 ml of Van Soest medium and substrate according to treatment design, 0.5g of glucose or 0.5g of glucose with 5mg of daidzein. The amount of daidzein we used in each fermentation bottle is normally present in about 1 to 5 g of soybean (Eldridge *et al*., 1983; Wang *et al*., 1994).

The cumulative gas production, the end concentration of lactate and VFA were detected to evaluate the effect of daidzein as well as age on the fermentative activity of intestinal microbiota. A *Lactobacillus*-specific PCR in combination with DGGE and a culture-based plate count method selective for lactic acid bacteria were used to investigate the effect of daidzein and age on the *Lactobacillus* community in the ileum and colon at different ages. All the procedures involving animals were conducted in accordance with the Chinese law on experimental animals.

2.2 Animals, inocula and incubation

Twelve healthy conventionally raised crossbred piglets (Yorkshire × Erhualian) of one litter were used as inocula donors. From neonate to 4 weeks of age (one week after weaning), piglets followed the general feeding strategy in China. This strategy involves creep feeding, (with in our study the creep feed from Agribrands Purina Nanjing Feedmill Co., Ltd). The main composition of creep feed is about 70.5 percent of maize, 20 percent of soybean meal, 5.5 percent of fishmeal and 4 percent of premix. This was supplied from 1 week of age until weaning at 3 weeks of age.

After euthanization at 1, 2, 3 and 4 weeks of age, the gastrointestinal tracts of individual animals were ligated at the portal and rectal side and immediately taken to the laboratory for further sampling. At the laboratory, chyme was surgically collected from ileum and colon of three piglets. To prepare ileal and colonic inocula for in vitro fermentation assays, respectively, digesta from three piglets from the respective intestinal compartment were immediately transferred into one container filled with CO_2, mixed and diluted with sterile Van Soest medium (1/10)(w/v) which had been pre-warmed to 39°C.

Five ml of inoculum was injected into each fermentation bottle. Each bottle contained 90 ml of Van Soest medium and substrate according to treatment design, and was incubated at 39°C for 48h. All material and media used were sterilized for 20 min at 121°C.

2.3 Sampling and analysis

After 48h fermentation, the pH value of each bottle was recorded, and approximate 10 ml of end liquids were sampled and divided into aliquots for downstream analyses, including total DNA extraction followed by 16S rRNA gene targeted PCR-DGGE and dilution PCR analysis, determination of the lactic acid (Zhang *et al*., 1997), VFA (acetate, propionate, and butyrate) (Qin, 1982) production, and plate count of lactic acid bacteria (LAB). For cultivation, 100 μl of the end liquid of each fermentation bottle was serially tenfold diluted in sterile water. Hundred μl of 10^3, 10^4 and 10^5 – fold dilutions were spread on de Man, Rogosa and Sharpe (MRS, Tidegene, China) agar plates, respectively, and incubated at 37 °C for 48h to determine colony forming units (CFU) of LAB.

The cumulative gas production was calculated based on the pressure and volume of head-space gases in a fermentation bottle recorded by using a manual pressure transducer during the entire fermentation period (Theodorou *et al*., 1994).

2.4 DNA isolation

Total DNA was extracted from digesta samples and fermentation liquids after 48 hrs by using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad. CA). Electrophoresis in agarose gels 1.2% (w/v) containing ethidium bromide was used to check the quality and yield of DNA visually.

2.5 PCR amplification

To investigate the *Lactobacillus*-like community by DGGE, PCR with primers Bact0011f (5'-AGA GTT TGA TCC TGG CTC AG-3') and Lab0677r (5'-CAC CGC TAC ACA TGG AG-3'), and nested PCR with primers Lab159f (5'-GGA AAC AGG TGC TAA TAC CG-3') and Univ0515rGC (5'-ATC GTA TTA CCG CGG CTG CTG GCA -3') by using 100-fold diluted products from previous amplification with Bact0011f and Lab0677r as template, were performed as described previously (Heilig *et al*., 2002).

By using serially diluted culture DNA isolated from culture fluid after 48h of fermentation as templates, dilution PCR with primers Lab0677r and Bact0011f was applied to semi-quantify lactobacilli (Koike *et al.*, 2003).

2.6 DGGE analysis of PCR amplicons and analysis of the DGGE gels

PCR products generated with Lab159f and Univ0515rGC were separated by DGGE according to the specification of Muyzer *et al*., using the Dcode system (Bio-Rad Laboratories, Hercules, Calif.). The gels were poured from the bottom by using a gradient maker and a pump (Econopump; Bio-Rad) set at a speed of 4.5ml/min, and gradients of 30% to 50% were used for the separation of the generated amplicons. Electrophoresis was performed during 16h at 85 V in a 0.5× TAE buffer at a constant temperature of 60°C. All $AgNO_3$ stained DGGE gels were scanned at 400 dpi. The similarity (Dice coefficients) of the DGGE profiles was calculated based on gel image analysis by using the software of Molecular Analyst (version 1.12, Bio-Rad).

2.7 Statistics

Differences between the treatments, intestinal compartment (inocula) and the interactions between them were tested for significance using two way ANOVA by SPSS system (V12.0). The statistical model used per age group was as follows:

$$Y=\mu + Si + Ij + (S*I)ij + \varepsilon ijk$$

Where Y is the parameter to be tested, μ is the overall mean, Si effect of the substrate I, Ij effect of the inoculum j. (S*I)ij denotes the interaction between substrate and inoculum. εijk is the error term.

3 Results

3.1 Spatio-temporal dynamics of the *Lactobacillus* community and the in vitro effect of daidzein

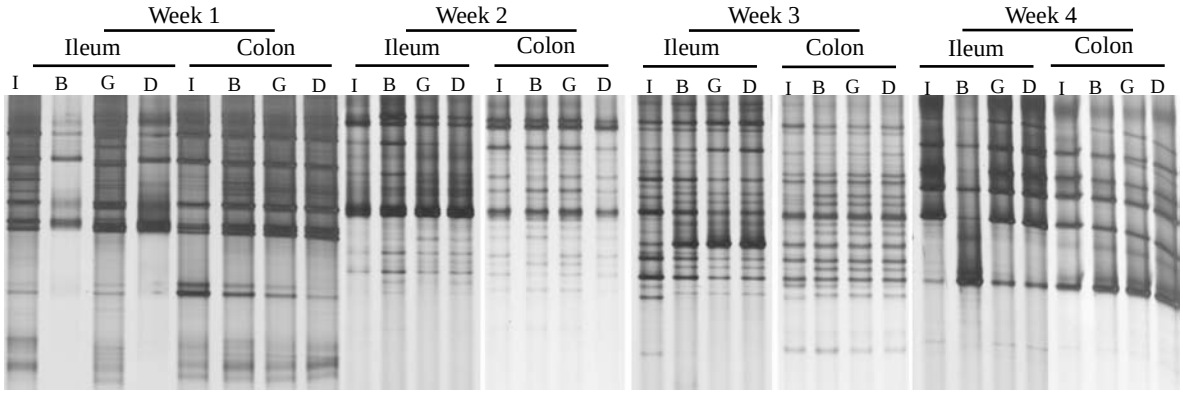

Figure 5.1 DGGE pattern of *Lactobacillus*-specific PCR amplicons derived from inocula and culture DNA after 48h fermentation. Ileal and colonic chyme from each three piglets euthanized at 1, 2, 3 and 4 weeks of age were used as inocula. I: Inoculum; B: Blank, Van Soest medium only; G: Glucose, medium with 0.5g of glucose; D: Daidzein, medium with 0.5g of glucose and 5mg of daidzein.

Table 5.1 Dice similarity coefficients (%) for comparison of *Lactobacillus* community profiles as affected by treatments and inocula. B: Blank, Van Soest medium only; G: Glucose, medium with 0.5g of glucose; D: Daidzein, medium with 0.5g of glucose and 50mg·L^{-1} of daidzein.

week	Ino	Ileum			Colon		
	Ile/Col	B/G	B/D	G/D	B/G	B/D	G/D
1	95.8	92.3	96.1	92.3	96.4	96.4	97.8
2	95.5	97.2	97.9	97.2	97.1	96.0	96.0
3	97.0	94.8	94.8	98.9	99.0	98.7	98.7
4	92.6	95.8	95.8	95.8	97.9	96.0	96.0

For the same intestinal compartment (inoculum), the DGGE patterns became more complex as piglets developed from 1 to 3 weeks (weaning time). After weaning, however, diversity decreased (Figure 5.1). Several new bands (i.e. new populations of lactobacilli) were observed at 3 weeks of age only, and disappeared again at week 4. For the same age of piglets, chyme from ileum and colon showed only slight differences in DGGE patterns (Figure 5.1) with similarity coefficients over 90% (Table 5.1). For each chyme, the three treatments showed similar DGGE patterns (Fig 5.1), with high similarity coefficients (Table 5.1). This suggests no apparent effect of daidzein on overall composition of the developing *Lactobacillus* community.

3.2 In vitro effects of daidzein on the number of lactobacilli and lactic acid bacteria

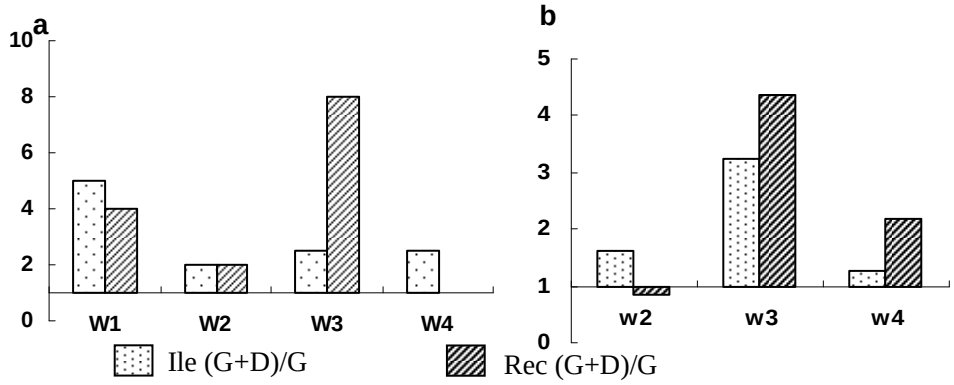

Figure 5.2 Ratio of the amount of *Lactobacillus* 16S rRNA gene copies (a) and ratio of the population of LAB (b) after 48h fermentation in glucose and glucose with 5mg of daidzein per bottle. (a) Results from dilution PCR with primers Lab0677r and Bact0011f; (b) Results from LAB plate count. G: Glucose, medium with 0.5g of glucose; D: Daidzein, medium with 0.5g of glucose and 50mg of daidzein.

Dilution PCR showed that daidzein increased the relative abundance of lactobacilli for most chymes irrespective of intestinal compartment and piglet age (Figure 5.2a), except for the colonic inoculum at 4 weeks of age. LAB plate count confirmed this effect of daidzein, except for the colonic inoculum at 2 weeks of age (Figure 5.2b).

CFU of LAB in ileal chyme increased as piglets grew from 1 to 3 weeks (weaning time) and then decreased slightly at week 4 (1.1E+09, 1.7E+09, 7.2E+09 and 4.3E+09 respectively). CFU in colonic chyme showed a similar increase from 1 to 3 weeks, but no

obvious decrease in week 4 (1.2E+09, 3.9E+09, 2.6E+10, 2.4E+10 respectively). Whereas CFUs of LAB in ileal and colonic chyme were at the same level in the first two weeks of life, CFU of LAB was tenfold higher in colonic chyme compared to ileal chyme in weeks 3 and 4.

3.3 Change of microbial gas production with age and the in vitro effect of daidzein

Except at week 1, there was no significant effect of daidzein on cumulative gas production. For week 1, daidzein decreased gas production. Except for week 3 chyme, the gut compartment of piglets significantly affected microbially produced gas yield (Table 5.2). More gas was produced with ileal inoculum compared to colonic inoculum in week 1. In week 2 and 4, however, significantly more gas was produced with colonic inoculum. The cumulative gas production by ileal inoculum decreased and that by colonic inoculum increased with age.

3.4 Effect of chyme source and age of piglets on concentrations of VFA and lactic acid and the in vitro effect of daidzein.

Daidzein significantly increased the concentration of TVFA after fermentation with week 3 chyme ($P<0.05$) (Table 5. 2). Propionate and lactate were increased irrespective of time, but there was no significant effect on acetate and butyrate. Significant effects of inoculum source, i.e. ileum vs. colon, were observed for concentrations of acetate and lactate ($P<0.05$ and $P<0.001$). For acetate, remarkably lower amounts were produced by ileal inocula from 1 to 4 weeks of age. For lactate, lower yields were obtained by colonic inocula. For propionate, higher yields were detected by ileal inocula from 1 to 3 weeks of age, however, lower on week 4. For TVFA, significantly higher yields were observed by ileal inocula at 1 week of age ($P<0.01$), and remarkably higher yields were obtained by colonic inocula at 3 weeks of age. Clear effects of age were observed for TVFA, individual VFA and lactate production. For ileal inocula, the concentration of TVFA and acetate decreased from 1 to 3 weeks of age and increased again at 4 weeks of age, while the concentration of propionate and lactate decreased in succession from 1 to 4 weeks of age. For colonic inocula, a similar change of the concentration of TVFA and acetate were also observed from 1 to 4 weeks of age. The concentration of propionate increased and lactate decreased with age

Table 5. 2. Cumulative gas production, VFA profile and lactate production after 48h of fermentation of glucose and glucose with daidzein using ileal and colonic chyme of piglets at different euthanizing time as inocula.

	GI Site	Substrat	CV	TVFA	Acet	Prop	But	Lact
W1	Ileum	G	225.35	3.59	1.75	1.50	0.34	0.24
		G+D	215.36	4.66	2.56	1.82	0.28	0.49
	Colon	G	172.72	2.90	1.75	0.97	0.18	0.02
		G+D	168.06	2.67	1.44	1.09	0.15	0.17
		SEM	7.72	0.29	0.15	0.12	0.03	0.06
	Main effect	Sub	0.030	0.305	0.267	0.210	0.259	<0.001
		Site	<0.001	0.008	0.028	0.004	0.003	<0.001
		Sub*Sit	0.365	0.131	0.028	0.530	0.608	<0.001
W2	Ileum	G	202.19	2.30	0.98	1.22	0.09	0.19
		G+D	205.36	2.64	0.99	1.59	0.06	0.33
	Colon	G	207.10	2.75	1.85	0.69	0.21	0.014
		G+D	223.89	2.83	1.53	1.13	0.17	0.018
		SEM	3.22	0.18	0.15	0.12	0.02	0.04
	Main effect	Sub	0.064	0.622	0.552	0.033	0.195	0.028
		Site	0.036	0.457	0.023	0.014	0.002	<0.001
		Sub*Sit	0.181	0.759	0.528	0.839	0.948	0.036
W3	Ileum	G	190.99	2.07	0.99	0.87	0.20	0.037
		G+D	181.27	3.15	1.37	1.49	0.28	0.061
	Colon	G	184.04	2.10	1.33	0.59	0.18	0.005
		G+D	189.99	3.13	1.62	1.30	0.21	0.006
		SEM	2.96	0.22	0.09	0.13	0.02	0.01
	Main effect	Sub	0.774	0.020	0.060	0.006	0.171	0.119
		Site	0.892	0.989	0.093	0.229	0.280	<0.001
		Sub*Sit	0.250	0.947	0.792	0.817	0.537	0.156
W4	Ileum	G	188.84	2.52	1.67	0.63	0.22	0.071
		G+D	195.78	3.23	1.66	1.40	0.17	0.098
	Colon	G	204.00	4.84	2.75	1.76	0.32	0.001
		G+D	207.28	5.28	2.41	2.60	0.27	0.006
		SEM	3.07	0.40	0.15	0.24	0.02	0.01
	Main effect	Sub	0.344	0.121	0.212	0.013	0.163	0.002
		Site	0.030	<0.001	<0.001	0.002	0.014	<0.001
		Sub*Sit	0.729	0.687	0.236	0.904	0.962	0.019

SEM = Standard error mean; CV=Cumulative gas volume (ml/100ml incubated liquid); Acet = Acetic acid (mmol/100ml); Prop = Propionic acid (mmol/100ml); But = Butyric acid (mmol/100ml); TVFA = Total volatile acid (mmol/100ml); lact = lactic acid (mmol/100ml); G: glucose; D: daidzein; W: week.

4 Discussion

The current study primarily aimed to investigate the in vitro impact of daidzein on compositional and functional aspects of the porcine intestinal *Lactobacillus* community during the nursing period. In addition, the differences in compositional and functional characteristics of lactobacilli between the foregut and hindgut of piglets at different euthanizing times during the first 4 weeks of life were assessed.

Group-specific PCR in combination with DGGE showed, for the same intestinal compartment, that *Lactobacillus* community patterns became more complex during the first 3 weeks (before weaning) and then less diverse after 4 weeks of age. For the same age of piglets, digesta from ileum and colon showed similar DGGE patterns. These results agree with our paralleled in vivo study (see chapter 3). In that study, the *Lactobacillus* communities in different GI tract compartments developed over time, showing a trend from simple to complex and return to simple after weaning. The *Lactobacillus*-like communities in the whole GI tract from duodenum to rectum remained relatively stable. No significant differences were observed between the foregut and the hindgut. In another study, comparative analysis of DGGE profiles with universal bacterial primers (Bact0968GC and Bact1401) also indicated changes in ileal and colonic community composition before and after weaning, in line with our study (Konstantinov *et al.*, 2006). It is well known now that the gut microbial community in monogastrics develops from simple to diverse and becomes established (Bauer *et al.*, 2006). In response to the shift of diet, health and the environment, microbiota composition of piglets transiently changes (Mackie *et al.*, 1999; Konstantinov *et al.*, 2004 and 2006). This property of the piglet microbiota probably makes it amenable to manipulation by probiotics and prebiotics.

In line with the development of the microbial community of piglets, apparent spatial and age differences with respect to the fermentation activity were also observed in this study. During the first week of life, the ileal microbiota produced more gas, more TVFA and more lactic acid than the colon microbiota. In this week, piglets only suckled maternal milk with lactose as primary carbohydrate. Lactose is a very well fermentable carbohydrate, and is primarily used by the foregut microbiota before it can reach the hindgut microbiota. For example, lactic acid bacteria counts in the ileal content of broiler were significantly increased by dietary supplement of 3.5% of dried whey (80% lactose)

(Samli *et al*., 2007). At slaughtering, we observed a relatively short and thin caecum and colon with only a very small amount of digesta present at week 1. This indicates the lack of sufficient substrate for hindgut microbiota enrichment. As piglets grow, the large intestine continuously becomes enlarged in length and diameter during week 2, 3 and after weaning at week 4. This is associated with ingestion of solid feed. The long transit time and abundant substrate make the large intestine a better niche of microbiota and becomes a heavily colonized region of the gastrointestinal tract (Williams, *et al*., 2001). This is in line with our observation that more gas and TVFA were produced in the colonic fermentations at 4 weeks of age.

The nature of bacterial interaction can be either antagonistic or synergistic. Head-to-head competition for limiting resources results in competitive exclusion between microbes. Only the more competitive populations remain and the community becomes less variable. Diversification can avoid this antagonistic competition and create positive feedback for some microbiota which would otherwise disappear or be marginalized. Microbes establish chemical food webs where the product of one microbe becomes the substrate for another (Ley *et al*., 2006). The production and utilization of lactic acid in the large intestine is such a synergistic relation between two groups of bacteria, lactate-producing and lactate-utilizing bacteria. Lactate-producing bacteria (lactic acid bacteria, LAB), mainly include lactobacilli, bifidobacteria, enterococci and streptococci. In the human and animal gut, they produce more than 50% of lactic acid of the total end products from carbohydrate fermentation (Ewing and Cole, 1994). In the rumen, it is well established that members of genera such as *Megasphaera*, *Selenomonas*, and *Veillonella* are capable of utilizing lactate and converting it largely to acetate and propionate, and in the case of *Megasphaera*, also butyrate (Counotte *et al*., 1981). Lactate-utilizing bacteria, isolated from human feces, can use lactate to produce butyrate as a major fermentation product (Duncan SH *et al*., 2004). A lactate-utilizing bacterium LB01, isolated from porcine feces, can use lactate to produce propionate and butyrate (Liu *et al*., 2007). Thus, lactate is seldomly detected in rumen or in human and animal feces under normal conditions. When high levels of lactate are found, it can be regarded as an indication for an unbalanced colonic microbiota. Lactate accumulates in colonic chyme from individuals who are suffering from ulcerative colitis, at concentrations up to 100 mM (Hove *et al*., 1994). Lactate has also been reported to accumulate in feces from individuals who have

undergone gut resections (short bowel syndrome) (Kaneko *et al*., 1997). In individuals with no apparent disease, however, colonic lactate is usually less than 5 mM. Similar results were observed also in this in vitro study. The final lactate concentration in colonic fermentation liquid was very low (0.1 mM). In contrast ileal fermentation liquid contained about 1.9 mM, about 20fold more than colonic chyme. For the piglet foregut, acidification by lactic acid bacteria can decrease the pH value of the foregut and gives protection to the host against most pathogens (Ewing and Cole, 1994).

Daidzein has been proven to exert beneficial effects on farm animals (Han *et al*., 2006). The gut microbiota plays a very important role in the metabolism of daidzein. Its end metabolite equol has a higher estrogenic potency in vivo (Setchell *et al*., 1988 and 2002; Joannou *et al*., 1995). It has been suggested that one reason for the observed individual variation of the effect of daidzein on humans is due to individual differences in gut microbiota composition (Rafii *et al*., 2003, 2004; Atkinson *et al*., 2004). Similar individual variations have also been observed in animal trials. This variation was mainly explained with the dependency of the estrogenic or anti-estrogenic effect of daidzein on animals on the hormone levels of the animal (Han *et al*., 2006). It is not clear to what extend the intestinal microbiota is involved in this individual variation, and in the direct effect of daidzein on the gut microbiota in pigs. In this study, for both chymes, the three treatments showed similar DGGE patterns. This suggested that daidzein had no apparent effect on the composition of the *Lactobacillus* community. Plate count of LAB, however, showed an increase in the number of lactic acid bacteria with daidzein, and dilution PCR showed an increase of the relative abundance of lactobacilli, irrespective of intestinal compartment and piglet age. As a consequence daidzein increased lactate production significantly. Propionate production was also increased with daidzein. Lactate can be the substrate for lactate-utilizing bacteria to produce propionate (Liu *et al*., 2007). These results suggested that daidzein at 50mg·L^{-1} has a positive effect on the fermentation activity, and on the lactate and propionate producing activity of porcine intestinal microbiota. Our previous research on rumen bacteria also revealed a direct effect of daidzein on their fermentation activity using *in vitro* techniques (Zhu *et al*., 2002). Daidzein at 5 and 10 mg·L^{-1} could significantly increase the proportion of propionate in total VFA when rumen samples from native goats were used as the inoculum. A similar pattern was observed with mixed rumen or faecal anaerobic fungi in gas production and

substrate degradation. Using a pure culture of a rumen anaerobic fungus, *Neocallimastix* sp., daidzein at 10 $mg \cdot L^{-1}$ and 20 $mg \cdot L^{-1}$ significantly increased cumulative gas production and dry matter (DM) loss. With mixed rumen fungi, daidzein at 10 mg/L also significantly affected gas production, although no significant difference was observed for DM loss. Daidzein can be metabolized by microorganisms, but daidzein and its metabolites which have estrogen-like activity are not a preferable growth substrate for microorganisms. The mechanism of the direct effect of daidzein on microbial growth and activity, however, is still unclear. Estrogenic receptor-like domains may exist on the cell walls of microorganisms. Tarry *et al*. (2005) confirmed that a yeast isolate of *C. albicans* contained a high-affinity estrogen-binding protein (EBP). Vaginal colonization by *C. albicans* was 8.6-fold greater in response to *in vivo* treatment with estradiol than with the comparable dose of diethylstilbestrol (DES), an efficacious mammalian estrogen receptor agonist. Although there is no direct evidence for intestinal microbiota, indirect evidence was obtained in studies with ruminants. In water buffalos fitted with permanent rumen- and intestine fistulas, it was demonstrated that injection of daidzein (500 mg/d, 12d) via a duodenum cannula could increase serum testosterone, rumen bacterial protein, ammonia nitrogen and total volatile fatty acids (VFA) (Chen *et al*., 1999). The level depends on the fluctuation of blood testosterone concentrations, which could enter the rumen with saliva or via the rumen epithelium (Yang *et al*., 1998). Daidzein may affect microbial activity and their metabolism by increasing the blood and rumen testosterone levels. It’s also possible that daidzein and its metabolites enter the rumen and affect microbes directly after absorption into blood and in consequence entering rumen via saliva and rumen epithelium, but the concentration of daidzein and its metabolites in saliva and rumen liquid were not detected in these studies.

In conclusion, the *Lactobacillus* community of ileal and colonic chyme develops over time. *Lactobacillus* diversity increases as piglets grow from 1 to 3 weeks (before weaning), but decreases after weaning. The fermentation ability of the ileal and colonic mictobiota are not the same. Daidzein does not affect *Lactobacillus* community composition in ileal and colonic chyme of piglets *in vitro*, but it significantly increased overall relative abundance of lactobacilli. The proportion of propionate in TVFA by all inocula, and lactate production by foregut inocula were increased remarkably by daidzein. These findings suggest that daidzein may have the potential for use as a prebiotic substance in

animal feed.

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CHAPTER 6

Modulation of the Gastrointestinal Bacterial Community of Weaning Piglets by Daidzein

This chapter has in part been published in:

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Abstract

The effect of daidzein on the intestinal bacterial communities of piglets was studied by 16S ribosomal RNA (rRNA)-based techniques. Three litters of piglets (Landrace-Yorkshire-Duroc) were selected. Within 3 litters, piglets were randomly allocated in two groups: control and treatment group. The piglets of the treatment group were fed 1 mg of daidzein as pure extract orally on day 7, 9 and 11. All piglets were weaned on day 21. One piglet from every litter of each group was randomly euthanized on day 14, 21, 24 and 35, respectively. The digesta of duodenum, jejunum, ileum, caecum, colon and rectum were collected, used for DNA extraction and subjected to microbiota fingerprinting by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments. The diversity of intestinal microbial communities of the piglets was assessed based on the Shannon's index as calculated based on number and relative intensity of DGGE bands. The 16S rRNA sequences of intestinal bacterial populations were analyzed. Piglets of the treatment group showed a higher bacterial diversity and more DGGE bands compared with animals of the control group on days 24, 35. Three DGGE bands observed in the control were absent in the treatment group profiles. Cloning and sequence analysis revealed that the corresponding populations were most closely related to *Clostridium thermocellum*, *Lactobacillus pontis* and *Streptococcus* sp., respectively. Three other DGGE bands were present only in the treatment group, and their corresponding sequences exhibited highest similarity to that of Butyrate-producing bacterium SL7/1, *Clostridium butyricum*, and *Ruminococcus obeum*, respectively. In conclusion, daidzein could increase the diversity of the piglets' intestinal bacterial communities, and change their composition after weaning.

Key words: daidzein; piglet; PCR-DGGE; intestinal microbial community

1. Introduction

Daidzein is naturally present in soybean and soy products. It is widely believed that the relatively low incidence of breast and prostate cancer in humans in China is mainly associated with the large quantity of consumption of soy products. The positive effects of daidzein on e.g. growth performance, immune system, and intestinal microbiota of farm animals in China have been recently reviewed (Han *et al*., 2006). The gut microbiota plays an important role in the metabolism of daidzein after ingestion by the host. Daidzein can be converted into dihydrodaidzein (DHD), *o*-desmethylangolensin (*o*-DMA) and equol. About one third to one half of human individuals can convert dietary daidzein into equol, others into DHD and/or *o*-DMA. Devoid of any gut microbiota, germ-free rats could not degrade daidzein (Schoefer *et al*., 2002; Setchell 2002a, 2002b; Bowey *et al*., 2003; Wang *et al*., 2005). Importantly, the estrogenic activity can be enhanced after daidzein is converted into more active metobolites by the gut microbiota (Rowland *et al*., 1999). Individual characteristics of gut microbiota composition may drive the fate of daidzein metabolism, and consequently could be the reason for the observed individual differences with respect to the effectiveness of daidzein treatment (Han *et al*., 2006).

On the other hand, several *in vitro* studies showed the direct effect of daidzein on the gut microbiota of human or pig. De Boever *et al*. (2000) studied the effect of a soygerm powder rich in β-glycosidic isoflavones on the fermentation pattern of the colon microbiota of healthy humans by using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The supplement of 2.5 g/d of soygerm powder, which provided 62.5 mg/d of isoflavone, promoted an overall increase of populations of *Enterobacteriaceae*, coliforms, *Lactobacillus* spp., *Staphylococcus* spp. and *Clostridium* spp., with a significant increase of the *Lactobacillus* spp. population. This study indicated that the consumption of soygerm powder may beneficially influence the gut microbiota. This beneficial effect could, however, not be completely attributed to isoflavones due to their low content in soygerm powder. We previously observed similar results in *in vitro* fermentation studies by using porcine intestinal chymes as inocula. 50 mg/L of daidzein significantly increased the number of lactobacilli (Yao et al., 2004).

To assess, whether daidzein beneficially affects the composition of the intestinal microbiota *in vivo*, this study aimed to investigate the effect of daidzein on the gastrointestinal bacterial community of piglets by using denaturing gradient gel

electrophoresis (DGGE) community fingerprinting in combination with cloning and sequence analysis of PCR-amplified 16S rRNA gene fragments.

2. Materials and methods

2.1. Animals and sampling

Three litters of piglets (Landrace-Yorkshire-Duroc) were selected. Piglets in each litter were randomly divided into two groups: control and treatment. Each piglet in the treatment group received 1 ml of pure daidzein extract (1000mg/L) in 1% of skimmed milk solution through oral administration at 7, 9 and 11 days of age. Daidzein (more than 97% of purity) was provided by the Key Laboratory of Animal Physiology and Biochemistry, Ministry of Agriculture of the People's Republic of China. The amount of daidzein we used, 1 mg for each piglet per day, is normally present in about 1 g of soybean (Eldridge *et al*., 1983; Wang *et al*., 1994). Piglets in the control group received 1 ml of skimmed milk solution at the same age. Piglets were weaned on day 21 and had no creep feed during lactation. On the day of weaning, sows were removed from the piglets, while piglets remained in the pens. Piglets were fed ad libitum with free access to water. The composition of the diet is shown in Table 6.1.

Table 6.1 Composition of the diet used for the animal trial

Ingredients	g/kg
Corn	525.0
Soybean meal	304.0
Wheat middlings	50.0
Fish meal(70.6% crude protein)	35.0
Whey meal	30.0
Soybean oil	15.0
Limestone	11.7
Dicalcium phosphate	15.0
Vitamin and mineral premix	10.0
Salt	2.5
L-Lysine	1.8

On day 7, 14, 21, 24 and 35, one piglet from each replicate was sacrificed, respectively. The digesta of stomach, duodenum, jejunum, ileum, caecum, colon and rectum were collected. The digesta samples were kept at -20°C. All the procedures involving animals were conducted in accordance with the Chinese law on experimental animals.

2.2. DNA isolation

Total genomic DNA was extracted from piglet digesta samples by a bead-beating method using a mini-bead beater (Biospec Products, Bartlesville, OK, USA) and followed by phenol-chloroform extraction as previously described (Zoetendal *et al*., 1998). Electrophoresis in agarose gels 1.2% (w/v) containing ethidium bromide was used to check the amounts of DNA visually.

2.3. PCR amplification

Bacterial specific primers U0968f-GC(5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and L1401r (5'-CGG TGT GTA CAA GAC CC-3') (Nübel *et al*., 1996) were used to amplify the V6-V8 regions of 16S rRNA genes. PCR was performed using the Taq DNA polymerase kit from Promega (Madison WI, USA). The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) using the following program as described previously (Zhu *et al*., 2003): 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 68°C for 40 s, and 68°C for 7 min last extension. Aliquots of 5μl were analyzed by electrophoresis on 1.2% agarose gels (w/v) to check the sizes and amounts of the amplicons.

2.4. DGGE

The amplicons obtained from the digesta DNA were separated by DGGE according to the specifications of Muyzer *et al*. (1993), using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed in 8% polyacrylamide gel 37.5:1 acrylamidebisacrylamide (dimensions 160×160×1 mm) using a 38-48% denaturing gradient (Muyzer et al., 1998). The electrophoresis was initiated by pre-running for 5 min at a voltage of 200 V, and subsequently run at a fixed voltage of 85V for 12 h at 60°C. The gel was stained with $AgNO_3$ and developed after completion of electrophoresis (Sanguinetti *et al*., 1994).

2.5. Analysis of the DGGE gels

DGGE analysis of all samples was repeated twice. All gels were scanned using a calibrated densitometer (GS 800, Bio-Rad), and analyzed using the software of Molecular Analyst/PC (version 1.61, Bio-Rad) to obtain densitometric curves. As a parameter for the structural diversity of the microbial community, the Shannon index of general diversity, *H'* (Shannon *et al*., 1963), was calculated according to methods of Konstantinov *et al*. (2003).

2.6. Cloning of the PCR-amplified products and sequence analysis

PCR was performed with a Taq DNA polymerase kit from Life Technologies using primers 8f and 1510r (Lane, 1991), which amplify the almost complete bacterial 16S rRNA gene. Amplification was carried out as described previously (Zoetendal *et al*., 1998). The PCR product was purified with the QIAquick PCR purification kit (Invitrogen, Shanghai, China) and cloned in *Escherichia coli* JM10, using the pMD18-T vector system (TaKaRa, Dalian, China). The colonies of ampicillin-resistant transformants were picked after overnight growth, transferred to Luria broth medium, and incubated at 37°C overnight. One hundred microlitres of the above cultures were centrifuged and suspended in 100 μl TE buffer. The solutions were then boiled for 5 min to lyse the cells. The cell lysates were used as templates in PCR reactions using vector-specific primers Sp6 and T7 to check the size of the DNA inserts. The plasmids with appropriately sized inserts (approximately 1.5 kb) were used to amplify the V6-V8 regions by PCR as described above. The amplicons were then compared with intestinal DNA-derived PCR products from the same samples in DGGE profiles. Amplicons with distinct bands on DGGE profiles were selected and sent for sequencing (Invitrogen). Similarity searches of the GenBank DNA database were performed with the BLAST Search tool (Altschul *et al.*, 1990).

2.7. Nucleotide sequence accession numbers

The sequences of the cloned 16S rRNA gene fragments amplified from pig rectal digesta samples were deposited in the GenBank database. The new sequences, with their accession numbers in parentheses, are: P1 (DQ238618), P2 (DQ238609), P3 (DQ238610), P4 (DQ238611), P5 (DQ238612), P6 (DQ238613), P7 (DQ238614), P8 (DQ238608), P9 (DQ238615), P10 (DQ238616), Y2 (DQ288680), Y5 (DQ288682), SC (DQ256404).

2.8. Statistical analysis

For statistical analysis calculations were made to determine number of DGGE bands, and the Shannon index of general diversity as described above. The data were expressed as the mean±SE. The Student's *t*-test was used to determine the statistical significance of the differences between mean values.

3. Results

3.1. Effect of daidzein on the diversity of the bacterial community of the gastrointestinal tract of piglets

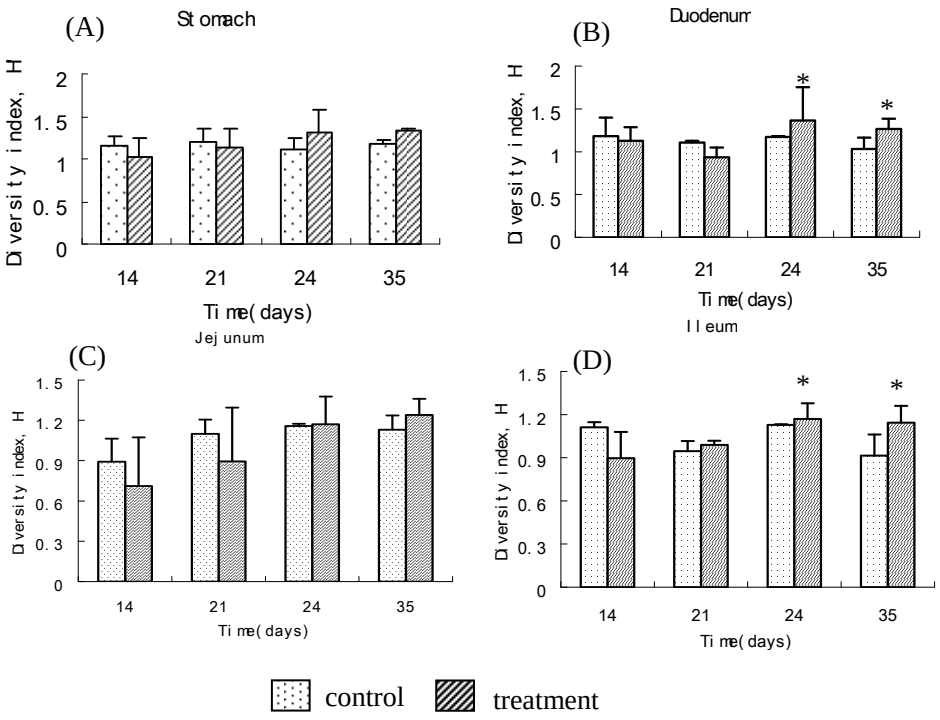


Figure 6.1. Analysis of diversity of DGGE profiles in time: days 14, 21, 24, 35. A: Stomach. B: Duodenum. C: Jejunum. D: Ileum.

Analysis of PCR-DGGE banding patterns using the Shannon's index of diversity (*H'*) was performed to measure the richness and evenness of gastrointestinal microbial communities based on band number and relative band intensity. Thus, *H'* was used as a parameter that reflects the diversity of the whole microbial community. Diversity was higher in the stomach of the control group compared with the treatment group before day 21. With time, however, the bacterial community in the stomach of the treatment group became more diverse, and the diversity of the treatment group (*H'*=1.26±0.13) was significantly higher

than that in the control group (*H*'=1.03±0.11) on day 35 (*P*<0.05) (Figure 6.1A.).

Before day 21, the bacterial community in the small intestine of the treatment group was less diverse than in the control group, albeit not significantly. After day 24, the bacterial community of the piglets in the treatment group became more diverse. The diversity of the bacterial community of duodenum and ileum of piglets of the treatment group was significantly higher than those observed for the control on day 24 (*P*<0.05) (Figure 6.1B, C, D).

The bacterial community in the caecum of the treatment group (*H*'=) was more diverse than the control on day 24 (*H*'=1.31±0.04 vs. 1.20±0.06) (*P*<0.05) and day 35.(*H*'=1.37±0.16 vs. 1.08±0.1) (*P*<0.05) (Figure 6.2A.).

The colonic bacterial community diversity of piglets from the treatment group (*H*'=1.34±0.05) was significantly higher than the control (*H*'=1.15±0.03) on day 35 (*P*<0.05) (Figure 6.2B.). The bacterial community diversity of rectal populations from the treatment group was higher than in piglets fed the control group after day 21, albeit not significantly (Figure 6.2C.).

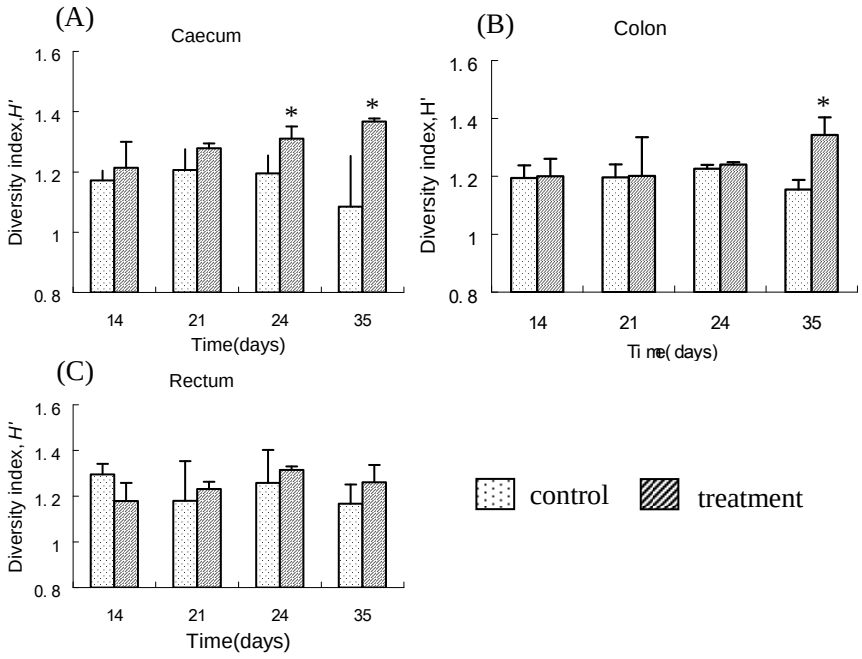


Figure 6.2. Analysis of DGGE banding patterns in time: days 14, 21, 24, 35. A: Caecum. B: Colon. C: Rectum.

3.2. Identification of dominant bacteria

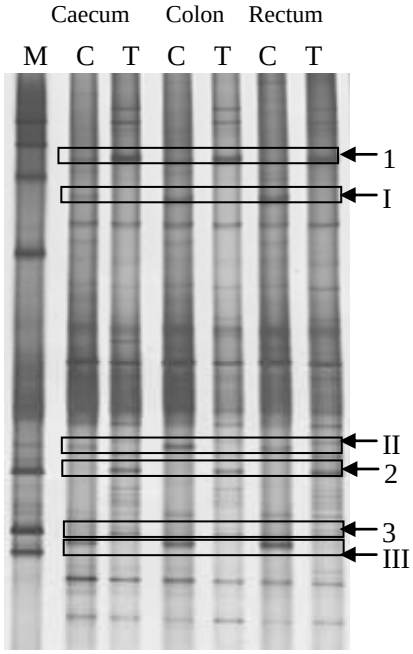
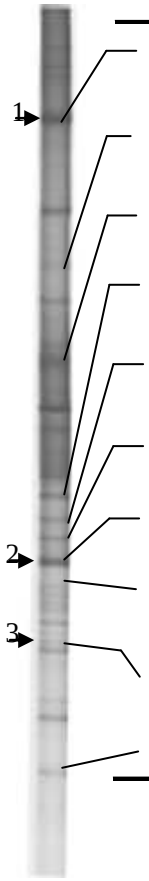


Figure 6.3. DGGE of PCR products of V6-V8 regions of 16S rRNA genes of piglet digesta samples from day 35. C: piglets on a control diet; T: piglets on diet with daidzein. Identity of populations represented by bands is indicated, using the most closely related sequences. 1: Butyrate-producing bacterium SL7/1; 2: *Clostridium.butyricum* (NCIMB8082); 3: *Ruminococcus obeum*; I: *Clostridium thermocellum* ATCC 27405 ctg206; II: *Lactobacillus.pontis* (LTH 2587); III: *Streptococcus* sp.

Table 6.2 Clones retrieved from the control group, with percentage of similarity to known sequences in GenBank and sequence length.

Clone	Closest relatives	similarity (%)	length (bp)
Y2	*Clostridium thermocellum* ATCC 27405 ctg206	87%	1309
Y5	*Lactobacillus.pontis* (LTH 2587)	89%	1026
SC	*Streptococcus* sp.	99%	1519



Clone	Closest relatives	Similarity (%)	Length (bp)
P1	Butyrate-producing bacterium SL7/1	95%	1399
P2	*Clostridium glycolicum*	97%	1458
P3	*Ruminococcus callidus*	93%	857
P4	*Clostridium disporicum*	95%	1372
P5	*Bacteroides capillosus*	93%	967
P6	*Clostridium* sp.	95%	1477
P8	*Clostridium butyricum* (NCIMB8082)	95%	1481
P7	*Clostridium viride*	94%	1477
P9	*Ruminococcus obeum* clone 1-4	96%	1501
P10	*Lactobacillus johnsonii* NCC 533	99%	1538

Figure 6.4. Identification of dominant bands in DGGE pattern of the V6-V8 region of rectal contents of piglets on day 35.

To study the effect of daidzein on the phylogenetic diversity of the predominant bacteria, the 16S rRNA gene pool from rectal samples of the control and treatment group was amplified, cloned and sequenced. The majority (10 out of 14) of the dominant bands showed less than 97% similarity with known sequences in the database (Figure 6.4). This indicates that most of the sequences were derived from new, as yet undescribed bacterial phylotypes.

Based on identical mobility within the gel, three common DGGE bands were present only in the large intestine of the control group, but were absent in the treatment group samples. These bands, indicated with I, II, and III in Figure 6.3, matched with clones Y2, Y5 and SC and were related to *Clostridium thermocellum* (87%), *Lactobacillus pontis* (89%) and *Streptococcus* sp. (99%), respectively. In addition, another three common DGGE bands

were enriched in the caecum, colon and rectum of the treatment group on day 35 (Figure 6. 3, bands indicated with 1, 2, 3). These bands matched with clones P1, P8 and P9 and were related to Butyrate-producing bacterium SL7/1 (95%), *Clostridium butyricum* (NCIMB8082) (95%), and *Ruminococcus obeum* (96%). These results suggest that the predominant bacteria appeared in the large intestine of the treatment group probably due to the presence of daidzein in the diet.

4. Discussion

Weaning is a major critical period of pig rearing because of increased susceptibility to gut disorders, infections and diarrhea (Lalles *et al*., 2004). Antibiotics have been used as animal growth promoter for decades, but many bacteria are becoming resistant to antibiotics (Smith *et al*., 2002). Thus there is an urgent need to find alternatives to in-feed antibiotics, especially after the full ban of antibiotic use as growth promoters within the European Union in 2006. Investigations over the past decade showed positive effects of the phytoestrogen daidzein on pigs. In studies with growing castrated male pigs, daidzein supplemented to diets at 5 mg/kg significantly increased weight gain by 59% ($P<0.01$), and the blood IGF-1 and testosterone levels were elevated by 51% and 18%, respectively (Guo *et al*., 2002). The doses of five mg/kg of daidzein supplemented to pregnant sows' diet could greatly increase the offspring's birth weight (Liu *et al*., 1996). By using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), the supplementation of 2.5 g/d of soygerm powder, which provided 62.5 mg/d of isoflavone, promoted an overall increase of populations of *Enterobacteriaceae*, coliforms, *Lactobacillus* spp., *Staphylococcus* spp. and *Clostridium* spp. (De Boever *et al*., 2000). By using a manual pressure transducer *in vitro* fermentation system, the supplementation with 50 mg/L of daidzein significantly increased the number of lactobacilli within piglet intestinal chymes (Yao *et al*., 2004). After incubation of daidzein *in vitro* with the feces from mice fed soy protein or casein diets, Tamura *et al*. (2002) found that the number of fusiform-shaped bacteria was significantly lower in the soy-isoflavone diet group than the control, whereas the number of lactobacilli was significantly higher. Together, these *in vitro* studies provided direct evidence for potential benefits of daidzein on the gut microbiota, and indicate the prebiotic potential of daidzein. Therefore, the primary aim of this study was to evaluate the *in vivo* effect of daidzein on intestinal microbiota of nursing piglets through

weaning.

Daidzein is a phytoestrogen, and is able to activate the mammalian estrogen receptor. Daidzein consequently exhibits estrogenic and anti-estrogenic activity *in vivo* based on the animal´s estrogenic level. This may be one of reasons that the effectiveness of daidzein on growth is more evident for male animals (Han *et al*., 2006), and reinforces the notion that the hormonal background of treated animals and the dosage of daidzein should be equally considered for experimental design. Compared to adults, piglets have a low level of endogenous estrogen. The umbilical cord serum estradiol levels of infants are about six-fold lower than the maternal serum estradiol levels at delivery and most of umbilical cord serum estradiol is of maternal origin (Nagata *et al*., 2006). A 14 mg/L supplement of genistein for 10 days to piglets within 48 h of birth was shown to inhibit cell proliferation in the jejunal crypts, with a concomitant tendency for reducing jejunal enterocyte migration in piglets. The ingested amount of genistein for each piglet per day during the experimental period ranged from 5 to 14 mg (Chen *et al*., 2005). In the same study, 1 mg/L supplement of genistein led to a slight increase of the average weight gain of piglets and jejunal disaccharidase activity, but no decrease in cell proliferation in the jejunal crypts. The ingested amount of genistein for each piglet per day in this experiment was from 0.36 to 1 mg. Considering these earlier studies, a supplementation of 1mg of daidzein per day was used in this study.

In this study, we applied cultivation-independent microbial community profiling to assess the effect of daidzein on intestinal microbiota composition. Indeed, comparison of the DGGE profiles of samples from animals of the treatment and control groups revealed significant differences after weaning. After weaning, the bacterial diversity in the piglets of the treatment group was higher than in the control piglets, especially in the caecal and colonic digesta of piglets. Most probably, this can not only be the direct effect of daidzein on gut microbiota. It can be assumed that at that time, the ingested daidzein has been completely eliminated from haemo-circulation for some days. Most of the daidzein and genistein is excreted within the first 24 h after dietary intake in human (Setchell, 2000). Ecological principles predict that host-driven selection for intestinal microbiota favors a stable community. Each microbial cell is under selection pressure in the gut. It is the current view that the immune system and the microbiota should be viewed as a co-evolved system. A driving force for evolution of the immune system is the need to accommodate a

diverse microbiota. In reverse, the immune system is the host's first line of contact with the microbiota in the gut and can be expected to play a role in shaping the microbiota (Ley *et al*., 2006). Zhang *et al*. (1995) reported that oral administration of daidzein in pregnant sows could affect immune function in the mammary organ as well as in the neonate piglet. Therefore, it is tempting to speculate that the observed differences in microbiota composition after weaning, long after daidzein treatment has been stopped, are based on the effect of daidzein on host piglet immune function, which then drives the development of the intestinal microbiota.

Decroos *et al*. (2005) obtained a mixed bacterial culture from human faecal samples that can transform daidzein into equol. DGGE fingerprinting analysis revealed that the culture consisted of four distinct phylotypes. Three of these 4 populations could be cultured, and they were identified as *Lactobacillus mucosae* EP12, *Enterococcus faecium* EPI1 and *Finegoldia magna* EPI3. Hur *et al*. (2002) identified a *Clostridium* sp. strain from human intestinal microbiota that converted daidzein to *o*-DMA. Interestingly, in this study, two predominant bands were observed exclusively in profiles in the treatment group, of which the corresponding clones showed 95% and 95% similarity to their closest relatives *Clostridium butyricum*, and a butyrate-producing bacterium, respectively. Both Butyrate-producing bacterium and *Clostridium butyricum* could produce butyrate (Zigova *et al*., 1999). Decroos *et al*. (2005) found that colonic fermentation products, particularly butyrate and propionate, stimulated equol production. This indicated that these predominant bacteria may be related to the daidzein metabolism. However, further studies are needed to evaluate the potential.

Konstantinov *et al*. (2003) found that *Ruminococcus* sp. was common in all DGGE profiles from pigs on a diet containing sugar beet pulp and fructooligosaccharides, but not in pigs that were fed a control diet lacking these ingredients. In this study, a dominant band was also found in the DGGE profile of pigs in the treatment group that was related to *Ruminococcus obeum* (96%). The genus *Ruminococcus* consists of anaerobic cocci, and the members of this group have been isolated previously from both human (Finegold *et al*., 1983) and pig faeces (Moore *et al*., 1987). These bacteria may play a role in the utilization of dietary fibres and have beneficial effect on the health of the host.

A predominant band in the DGGE profiles of the control was related to *Streptococcus* sp. (99%). This population only appeared in the large intestine of the control group, but

disappeared in the treatment group. Verdrengh *et al*. (2004) examined the potential antibacterial activities of daidzein, and the results showed that the growth of *Streptococcus aureus* was clearly inhibited by daidzein. Hence, our study confirms that that daidzein can exert potent antibacterial properties.

In conclusion, this *in vivo* study has demonstrated the effect of daidzein on intestinal microbiota of weaning piglets, including the stimulation, but also suppression of specific microorganisms after weaning. Populations with high similarity to known butyrate-producing bacteria were enriched, indicating the prebiotic potential of daidzein.

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CHAPTER 7

16S Ribosomal RNA-based Methods to Monitor Changes in the Hindgut Bacterial Community of Piglets after Oral Administration of *Lactobacillus sobrius* S1

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Abstract

Changes in the composition of microbiota in the hindgut of piglets were studied after oral administration of *Lactobacillus sobrius* S1, using molecular techniques based on 16S ribosomal RNA (rRNA) genes. Six litters of neonatal piglets were divided randomly into control group and treatment group. At 7, 9, and 11 days of age, piglets in the treatment group orally received a preparation of *L. sobrius* S1. At 7, 14, 21(weaning), 24 and 35 days of age, one piglet from each litter was sacrificed and digesta samples of hindgut were collected. V6-V8 and V2-V3 variable regions of the total bacterial and *Lactobacillus*-specific 16S rRNA gene pool, respectively, were amplified by PCR and analyzed by denaturing gradient gel electrophoresis (DGGE). DGGE analysis for all bacteria showed that several populations present in the hindgut of piglets, represented by far-migrating bands, disappeared after weaning. Sequence analysis indicated that most of these bands corresponded to *Lactobacillus* spp. This trend was also confirmed by quantitative real-time PCR. Drastic changes of *L. amylovorus* and *L. sobrius* in total *Lactobacillus* populations were also observed in the colon of piglets around weaning, as monitored by *Lactobacillus*-specific PCR-DGGE. Comparison of DGGE profiles between control and treatment groups revealed a specific band related to *Clostridium disporicum* that was found in treatment group on day 14. On day 35, a specific band appeared only in the control group, representing a population most closely related to *Streptococcus suis* (99%). Species-specific real-time PCR revealed that the population of *L. sobrius* declined apparently in the colon of piglets after weaning, while it tended to backer-establish earlier after oral administration of strain S1.

Key words*:* DGGE; *Lactobacillus*; piglet; hindgut; bacterial community

1. Introduction

The hindgut of the pig harbors a dense and metabolically active microbiota. This is comprised primarily of bacteria, which have a profound influence on nutritional, immunological, and physiological processes in the host. Recently, more attention has been paid to studies of the intestinal bacterial community, notably because of the urgent need to replace antibiotics as growth promoters in animal production (Konstaninov *et al*., 2003). In the past, the microbial ecology of gastrointestinal tract ecosystems was not well understood due to the inadequacy of classical, culture-dependent microbiological methods. Recently, cultivation-independent approaches, such as DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments have been increasingly employed in the study of complex bacterial ecosystems as found in the mammalian intestine (Simpson *et al*., 1999; Zhu *et al*., 2000; Favier *et al*., 2003; Plant *et al*., 2003; Konstantinov *et al*., 2004) and have been demonstrated to be a powerful tool for monitoring bacterial community shifts following environmental changes (Zoetendal *et al*., 2004). Moreover, recently introduced real-time PCR assays allow quantitative analysis of specific microbial groups and species in complex ecosystems (Suzuki *et al*., 2000; Konstantinov *et al*., 2006a).

Classical methods used to study bacterial populations in the cecum, colon, and feces of pigs have revealed a wide range of characteristic genera, including *Lactobacillus*, *Streptococcus*, *Peptococcus*, *Eubacterium*, *Clostridium*, *Bifidobacterium* and *Bacteroides* (Stewart 1997). Particular interest is focused on the *Lactobacillus* and *Bifidobacterium* populations within the gastrointestinal tract of the piglet, due to their proved benefits for gut function and health (Vaughan *et al*., 1999). Some studies have investigated the changes in the fecal bacterial community of piglets after introduction of a probiotic strain by using molecular techniques based on the 16S rRNA gene (Simpson *et al*., 2000; Walter *et al*., 2000). However, fecal samples can not reflect the situation in the intestinal tracts of animals completely (Zoetendal *et al*., 2002).

Lactobacillus sobrius S1 was isolated from piglet's jejunal digesta in our laboratory and was shown to have properties of an effective probiotic organism (Wu *et al*., 1999). Strain S1 is considered a rapidly growing strain which can 1) produce large amounts of lactic acid, 2) secrete bacteriocin-like products, and 3) exerts a strong inhibitory effect on *E. coli* and *Staphylococcus aureus*. A feeding trial also demonstrated that oral administration of a preparation made from this strain could reduce the incidence of diarrhea in suckling

piglets (unpublished data). To gain insight into the underlying mechanism of this effect, the present study investigated the changes of bacterial community composition in the hindgut of piglets during the sucking and weaning period and how these were affected by oral administration of strain S1.

2. Materials and methods

2. 1. Bacterial strain culture

Lactobacillus sobrius S1, isolated from piglet's jejunal digesta, was grown in MRS medium (Mann *et al.*, 1960) at 37°C for 12 h (late-logarithmic phase). Cultures of the strain were centrifuged at 3000×*g* / 10 min / 4°C, re-suspended with 10% skimmed milk solution, and then stored at -20°C. This preparation was diluted 10-fold with sterile water after dissolved, and used for the animal experiment, its count was determined by incubating on agar MRS medium for 24 h.

2. 2. Piglet experimental design

Table 7.1. Composition of the diet used for weaned piglets as fed-basis

Ingredients	g / kg	Nutrients parameters	
Corn	525.0	ME(MJ/Kg)	13.37
Soybean meal	304.0	CP(%)	22.09
Wheat middlings	50.0	Ca(%)	0.97
Fish meal	35.0	Available P(%)	0.51
Whey powder	30.0	Lysine(%)	1.31
Soybean oil	15.0		
Limestone	11.7		
Calcium hydrogen phosphate	15.0		
Vitamin and mineral premix[a]	10.0		
Salt	2.5		
L-Lysine	1.8		

[a]This mineral and vitamin premix (1%) supplies per kg diet as follow: VA 11 000 IU, VD3 (cholecalciferol) 1 000 IU, VE (tocopherol) 16 IU, VK1 (phylloquinone) 1mg, VB1 (thiamin) 0.6 mg, VB2 (riboflavin) 0.6 mg, d-pantothenic acid 6 mg, VB3 (nicotinic acid) 10 mg, VB12 (cyanocobalamin) 0.03 mg, folic acid (folacin 0.8 mg, VB6 (pyridoxine) 1.5 mg, choline 800 mg, Fe 165 mg, Zn 165 mg, Cu 16.5 mg, Mn 30 mg, Co 0.15 mg, I 0.25 mg, Se 0.25 mg.

Six litters of neonatal piglets (8-11 piglets in each litter) from a commercial maternal-line herd (Landrace-Yorkshire-Duroc) were randomly divided into two groups: control and treatment, each with three litters (replicates). Each piglet in the treatment group received one ml of strain S1 preparation (5×10^9 CFU·ml^{-1}) through oral administration at 7 days of age, 2 ml at 9 days, and 3 ml at 11 days. Each piglet in the control group received the same volumes as skimmed milk at the same age. Creep feed was not provided to piglets during the sucking period. Piglets were weaned at 21 days of age. On the day of weaning, sows were removed from the piglets, while piglets remained in the nursing pens. Piglets were fed ad libitum with free access to water. The diet (Table 7. 1) did not contain any antibiotics or acidifiers. All the procedures involving animals were conducted in accordance with the Chinese law on experimental animals.

2. 3. Sampling

Two diarrhoeal animals were registered in the control group during the course of animal experiment, while no unhealthy piglets were found in the treatment group. The diarrhoeal piglets were then not used for gut microbiota analysis. On day 7, 14, 21, 24 and 35, one piglet from each replicate was sacrificed, respectively. For molecular analysis, samples of homogenized cecal and colonic digesta (2-3 g) were collected and stored at –20°C until analysis.

2. 4. DNA isolation and PCR amplification

Total bacterial DNA was extracted from each sample (0.5 g) according to a bead-beating method using a mini-bead beater (Biospec Products, Bartlesville, OK, USA) and followed by phenol-chloroform extraction (Zoetendal *et al*., 1998). DNA was then precipitated with ethanol and pellets were dissolved in 50 μl of TE.

All PCR primers used in this study are listed in Table 7. 2. Primers U968-GC and L1401 were used to amplify the V6-V8 variable regions of the bacterial 16S rRNA gene (Nübel *et al*., 1996). PCR was performed with the Taq DNA polymerase kit from Promega (Madison, WI, USA). The samples were amplified in a T1 Whatman Biometra (Gŏttingen, Germany) using the following program: 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 68°C for 40 s, and 68°C for a final 7 min extension. To investigate the *Lactobacillus*-specific colonic bacterial community by DGGE, a specific nested-PCR approach was chosen (Heilig *et al*., 2002). For the initial amplification, Bact0027 (Kane *et*

al., 1993) and Lab0677 (Heilig *et al*., 2002) primers were employed, using the following cycling conditions: predenaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 66°C for 20 s, and 68°C for 40 s; and a final extension at 68°C for 7 min. The PCR products were then used as templates to amplify the V2-V3 regions of 16S rRNA gene in a nested PCR with Lab0159 (Heilig *et al*., 2002) and Uni0515-GC (Lane 1991). The cycling program was identical to the one used for the amplification of the V6 to V8 regions of the 16S rRNA gene. Aliquots of 5 µl PCR products were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing ethidium bromide to check size and amount of the amplicons.

Table 7.2. List of primers used in this study

Primer	Sequence 5′−3′	Reference
U968-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAC GGGGGGAACGCGAAGAACCTTAC	Nübel 1996
L1401	CGGTGTGTACAAGACCC	Nübel 1996
8F	CACGGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCAG	Lane 1991
1510R	GTGAAGCTTACGGCTACCTTGTTACGACTT	Lane 1991
T7	TAATACGACTCACTATAGG	Promega
Sp6	GATTTAGGTGACACTATAG	Promega
Bact0027	GTTTGATCCTGGCTCAG	Kane 1993
Lab0677	CACCGCTACACATGGAG	Heilig 2002
Lab0159	CGGTATTAGCACCTGTTTCC	Heilig 2002
Uni0515-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAC GGGGGGATCGTATTACCGCGGCTGCTGGCA	Lane 1991
LAC1	AGCAGTAGGGAATCTTCCA	Walter 2000
L-*-OTU171-00 77-a-S-2	ACTTCGGTAATGACGTTG	Konstantinov 2005
Bact1369	CGGTGAATACGTTCYCGG	Suauki 2000
Prok1492	GGWTACCTTGTTACGACTT	Suauki 2000

2. 5. DGGE analysis

DGGE was employed to separate the amplicons according to the specifications of Muyzer *et al* (1993), using a Dcode TM system (Bio-Rad, Hercules, CA, USA). DGGE was performed in 8% polyacrylamide gels containing 37.5:1 acrylamide-bisacrylamide using a 38 to 51% denaturing gradient for separation of PCR products obtained with primers U968-GC and L1401, whereas gradients of 30 to 45% were employed for the separation of the Lab0159 and Uni0515-GC generated amplicons. The electrophoresis was initiated

by pre-running for 10 min at a voltage of 200 V, and subsequently performed at a fixed voltage of 85 V for 12 h at 60°C. The gel was stained with 0.2% $AgNO_3$ after completion of electrophoresis (Sanguinetti *et al*., 1994).

2. 6. Analysis of DGGE gels

DGGE gels were scanned using GS-800 Calibrated Densitometer (Bio-Rad) and analyzed using the software of Molecular Analyst 1.61 (Bio-Rad). Similarities between DGGE profiles were determined by calculating a band similarity (Dice) coefficient, *SD*. $SD = 2n_{AB} / (n_A + n_B)$, where n_A is the number of DGGE bands in lane 1, n_B represents the number of DGGE bands in lane 2, and n_{AB} is the number of common DGGE bands. The Shannon index of general diversity, *S*, was calculated as a parameter for the structural diversity of the bacterial community (Konstantinov *et al*., 2003; Shannon and Weaver 1963). *S* was calculated using the following function: $S = -\sum P_i \log P_i$, where P_i is the importance probability of the bands in a lane. The importance probability, P_i, was calculated as: $P_i = n_i / H'$, where n_i is the height of a peak and H' is the sum of all peak heights in the densitometric curve.

2. 7. Cloning of the PCR amplified products and sequence analysis

Almost complete bacterial 16S rRNA genes from piglet hindgut samples (cecal sample of piglet in litter 6 on day 21; colonic sample of piglet in litter 1 on day 35) were amplified by PCR with primers 8F and 1510R (Lane 1991). PCR products were purified using Invitrogen PCR product purification kit (Invitrogen, Shanghai, China) and cloned in *E. coli* JM109 using the pGEM-T vector system (Invitrogen). Clonal colonies of ampicillin-resistant transformants were picked after overnight growth, transferred to Luria broth medium, and incubated at 37°C overnight. One hundred microlitres of the above cultures were centrifuged and suspended in 100 μl TE buffer. The solutions were then boiled for 5 min to lyze the cells. The cell lysates were used as templates in PCR reactions using pGEM-T specific primers Sp6 and T7 to check the size of the DNA inserts. The plasmids with appropriately sized inserts (approximately 1.6 kb) were used to amplify the V6-V8 regions by PCR as described above. The amplicons were then compared with intestinal DNA-derived PCR products from the same samples in DGGE profiles. Amplicons corresponding to interestin bands on DGGE profiles were selected and sent for sequencing (Invitrogen, Shanghai). Homology searches of the GenBank DNA database

were performed with the BLAST Search tool (Altschul *et al*., 1990).

2. 8. Real-time PCR assay for quantification of total bacteria, *lactobacilli* and *L. sobrius* in the colon of piglets

Real-time PCR was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, the Netherlands) as described previously (Konstantinov *et al*., 2005a). A reaction mixture (25 µl) consisted of 12.5 µl of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set and 5 µl of the template DNA. The amount of DNA in each sample was determined in triplicate, and the mean values were calculated. A standard curve was generated by using the serially diluted 16S rRNA gene amplicons obtained from *L. sobrius*. Universal primers, Bact1369 and Prok1492 (Suzuki *et al*., 2000), were used to estimate the total number of copies of the bacterial 16S rRNA gene in each sample. PCR was performed with an initial denaturation step of 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. Species-specific primer L-*-OTU171-0077-a-S-2 (Konstantinov *et al*., 2005a) combined with primer Lab0159 was used for the quantification of *L. sobrius* with the following conditions: an initial DNA denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and primer annealing and extension at 62.5 °C for 45 s. Total lactobacilli were quantified using the combination of forward, LAC1 (Walter *et al*., 2000), and reverse primer, Lab0677, in a cycling programme where after the initial denaturation 95 °C for 3 min, 40 cycles were applied at 95 °C for 30 s, and binding and extension at 60 °C for 1 min.

2. 9. Nucleotide sequence accession numbers

Thirteen sequences of hindgut bacterial 16S rRNA gene clones were obtained, and have been deposited in the GenBank database under accession numbers: DQ238609, DQ238611, DQ238615, DQ238616, DQ256404, DQ318869, DQ318870, DQ318871, DQ318873, DQ318875, DQ480422, DQ487214 and DQ487215.

2. 10. Statistical analysis

Differences between the treatment and control groups were tested for significance with an ANOVA program using the statistical software SPSS10.0. All values are expressed as means of replicates (three litters), treating one piglet from one litter as a single replicate

for each day. The statistical model used per age group was as follows:

$Y=\mu + Ti + Aj + (T*A)ij + \varepsilon ijk$

Where Y is the parameter to be tested, μ is the overall mean, Ti effect of the treatment I, Aj effect of the age j. (T*A)ij denotes the interaction between treatment and age. εijk is the error term.

3. Results

3. 1. Analysis of bacterial diversity in hindgut samples using PCR-DGGE

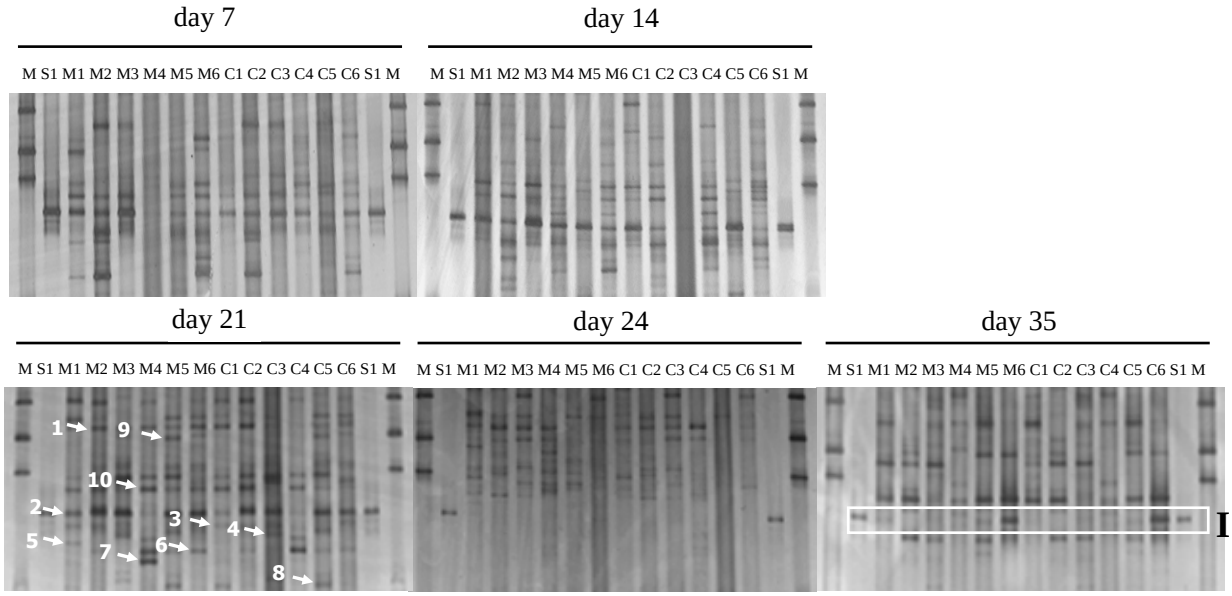

Figure 7.1. DGGE profiles from far-migrating bands corresponding bacteria in hindgut of piglet. M, Marker; S1, *Lactobacillus* sp. strain S1; M1-3 and M4-6, caecal samples from control (Litter 1-3) and treatment (Litter 4-6) groups, respectively, C1-3 and C4-6, colonic samples in control and treatment groups, respectively. Bands indicated with numbered arrows in gels of day 21 were identified as shown in table 3. Frame I, bands corresponding to strain S1.

DGGE profiles of PCR products of V6-V8 regions of 16S rRNA genes from piglet hindgut samples revealed remarkable differences between pre and post-weaning. Especially, amplicons migrating to the lower part of the gel were predominant in the hindgut of pre-weaning piglets, but did no longer belong to the abundant microbiota after weaning, and were even not restored until day 35 (Figure 7. 1). To identify the initially pre-dominant populations, the 16S rRNA genes from hindgut samples on day 21 were

amplified, cloned and sequenced, indicating that most of these populations belonged to *Lactobacillus* spp. (Figure 7. 1, arrows 1-10; Table 7. 3). We also found that the band that shared the same migration position with strain S1 on the gel corresponded to a population most closely related to *L. sobrius.*

Table 7.3. Identification of bacterial phylotypes corresponding to far-migrating bands observed in the hindgut of piglets

No.	Closest known species found in the GenBank database	Length (bp)	Sequence accession numbers	Similarity (%)
1	*Lactobacillus vaginalis*	1502	DQ318870	99%
2	*Lactobacillus sobrius*	1528	DQ487214	99%
3	*Lactobacillus johnsonii*	1538	DQ238616	99%
4	*Lactobacillus amylovorus*	1501	DQ318869	99%
5	*Lactobacillus delbrueckii subsp.*	1522	DQ318871	98%
6	*Lactobacillus pontis*	1521	DQ318875	99%
7	*Lactobacillus delbrueckii subsp.*	1290	DQ480422	97%
8	*Lactobacillus amylovorus*	1501	DQ318873	97%
9	*Ruminococcus obeum*	1501	DQ238615	96%
10	*Lactobacillus reuteri*	1525	DQ487215	99%

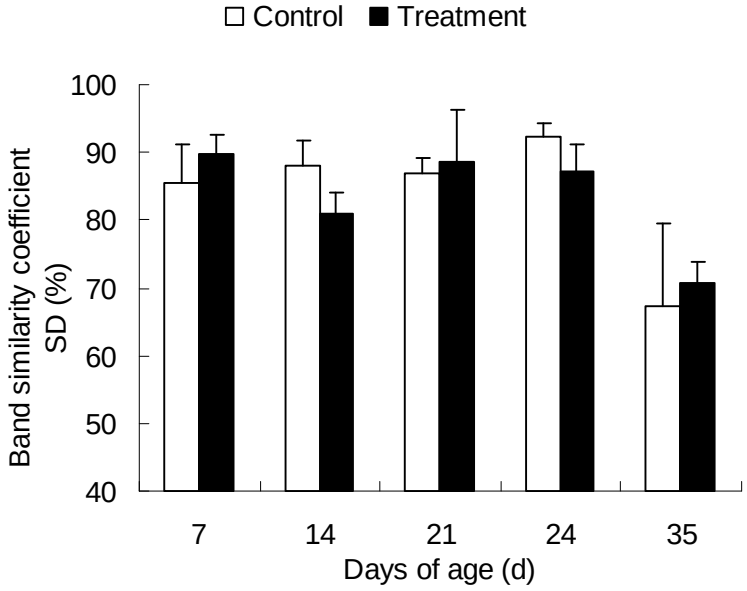

Figure 7.2. Dice coefficients for comparison between cecal and colonic samples of piglets in control and treatment groups. Bars indicate the standard deviation between the DGGE profiles from piglets in the same group.

To obtain an objective interpretation of the electrophoretic patterns of the control and treatment groups, the samples were subjected to a numerical analysis based on the Dice similarity coefficient, followed by cluster analysis. Cluster analysis revealed that there were no major changes between the two groups. Except day 35, other samples from the same age formed a cluster with similarity indices above 70%. Dice coefficient between cecal and colonic samples was also compared as shown in Figure 2. Statistical analysis showed that the similarity coefficient between cecal and colonic samples of piglets on day 35 either in control or in treatment groups was significantly lower than those in other ages ($P < 0.05$).

Shannon diversity indices were calculated to compare the diversity of the dominant bacterial microbiota in the hindgut of piglets from control (skim milk) and treatment (S1 preparation) groups. Statistical analysis showed that the index was not significantly different between the two groups neither in cecum nor in colon ($P = 0.597$, 0.302 respectively, date not shown). However, for both groups, bacterial diversity in the colon tended to decline abruptly three days after weaning, while this effect was not observed in cecum. Statistical analysis also showed that there was no difference in bacterial diversity between cecal and colonic samples in each age (data not shown).

3. 2. Identification of specific bands in DGGE profiles from control and treatment groups

A more detailed comparison of DGGE profiles between two groups revealed that a specific band was found on day 14 in the treatment group, clearly visible in M5, M6, C4, C6, and faintly visible in M4 and C5, but not visible in the control group (Figure 7. 3, indicated with I). Sequence analysis showed 95% similarity to *Clostridium disporicum* (GenBank accession number DQ238611). On day 35, a specific band, indicated with II (Figure 7. 3), appeared exclusively in the control group, and was identified to correspond to a population most closely related to *Streptococcus suis* (DQ256404, 99%). Also on day 35, bands corresponding to the probiotic strain S1 were visible for treatment samples M6 and C6 and faintly visible in M5 and C5 samples, but not visible in controls (Figure 7.1 frame I).

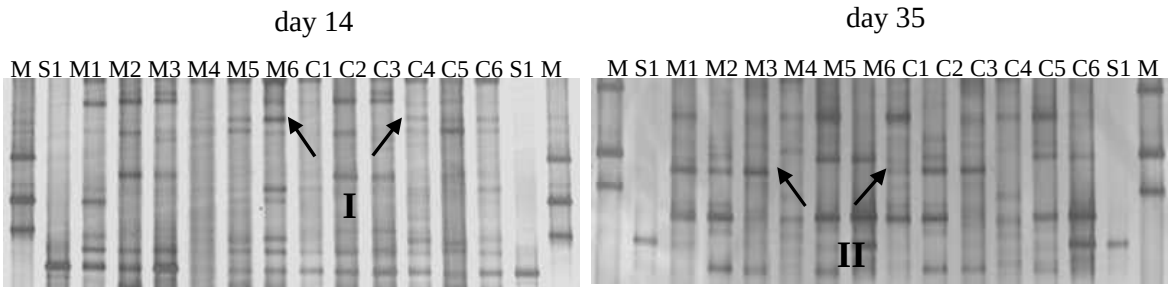

Figure 7.3. Identification of bands exclusively detected in DGGE profiles of control or treatment samples, respectively. Sample identifiers as in Fig. 7.1.

3. 3. Analysis of Lactobacillus diversity in colonic samples using PCR-DGGE

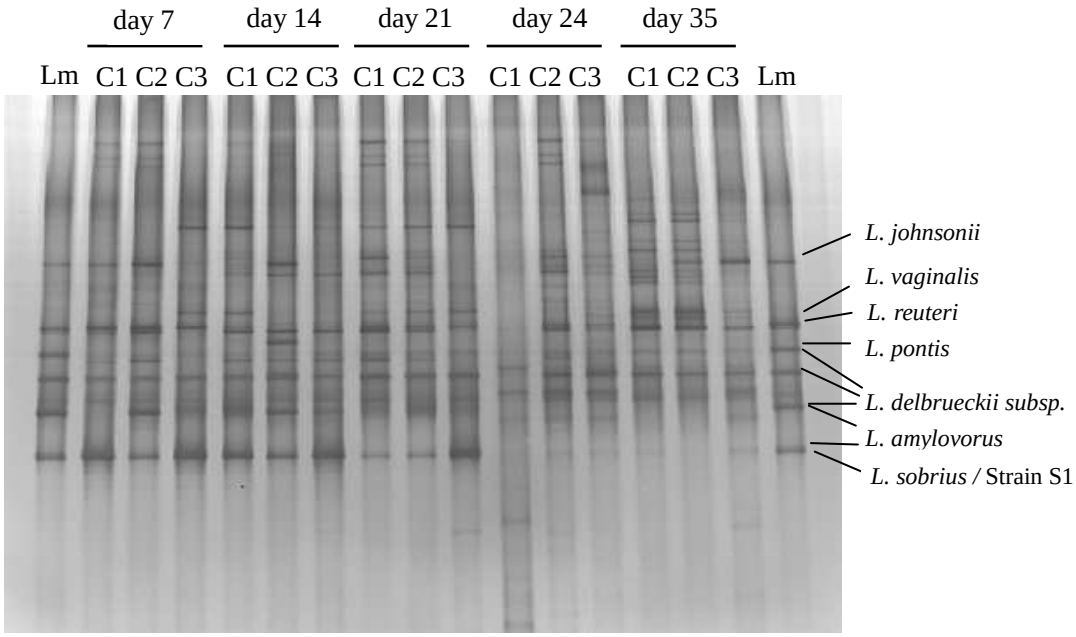

Figure 7.4. DGGE analysis of amplicons obtained from colonic samples by nested PCR with primers Lab-0159 and Uni0515-GC. The dominant fragments in *Lactobacillus*-like patterns were identified by the clones corresponding to *L. johnsonii*, *L. vaginalis*, *L. reuteri*, *L. pontis*, *L. delbrueckii* subsp,, *L. amylovorus* and *L. sobrius*. Lm, mixture of nine *Lactobacillus*-like clones.

Although the amplification of the V6 to V8 regions using bacterial universal primers allowed the visualization of the major differences between pre- and post-weaning samples, it yielded poor resolution of *Lactobacillus* populations. Therefore, specific amplification of the 16S rRNA gene pool corresponding to the colonic *Lactobacillus* community, in

combination with DGGE analysis using *Lactobacillus*-like clones from the hindgut of piglets as markers, were employed to monitor the dynamics of *Lactobacillus* populations (Fig. 7. 4). Bands related to *L. reuteri* and *L. delbrueckii* subsp. were consistently found in the samples from most piglets during the age of 7 to 35 days, while *L. amylovorus* and *L. sobrius* disappeared from the predominant *Lactobacillus* community in the colon of weaned piglets.

3. 4. Quantitative real-time PCR analysis of the predominant microbiota in colonic samples of piglets

Table 7.4. Quantitative real-time PCR analysis of total bacteria, lactobacilli and *L. sobrius* in porcine colonic samples [Lg (copies/g wet weight)]

Item	Bacteria		Lactobacilli		*L. sobrius*	
	Control	Treatment	Control	Treatment	Control	Treatment
Day7	10.94	10.98	9.43	9.52	8.44	8.59
Day14	11.09	11.32	9.51	9.68	8.42	8.74
Day21	10.81	11.20	9.52	9.57	8.49	8.41
Day24	10.65	10.92	6.93	7.09	6.10	6.58
Day35	10.65	11.05	8.28	8.95	6.83	8.45
SEM	0.24	0.22	1.17	1.21	1.19	1.08
Age	0.13		< 0.001		< 0.001	
S1 treatment	0.02		0.376		0.089	
Age*S1 treatment	0.592		0.945		0.424	

Counts are expressed as mean of replicates (three litters), treating one piglet from on litter as a single replicate for each day.

Since drastic changes were observed in bacterial community composition between pre- and post-weaning piglets, real-time PCR was used to quantify total bacteria, lactobacilli and *L. sobrius* in colonic samples of piglets in treatment and control groups (Table 7. 4). The assay showed that the number of total *Lactobacilus spp.* and *L. sobrius* 16S rRNA gene copies both in the control and treatment groups showed an apparent fluctuation from day 7 to 35 although there were no obvious changes in the density of total bacteria. In general, the numbers of total *Lactobacilus spp.* and *L. sobrius* declined significantly after weaning ($P < 0.05$). Except total bacteria on day 21, no significant difference was found

between treatment and control groups. However, *L. sobrius* 16S rRNA gene copies in treatment group tended to be higher than in control group ($10^{8.45}$ vs $10^{6.83}$) on day 35. In particular, this number in samples from litter 6 was high to $10^{9.43}$ which was consistent with the results of DGGE profiles (Figure 7.1 frame I).

4. Discussion

Weaning stress can destroy the balance of intestinal microbiota. It is generally accepted that lactobacilli are important to maintain good intestinal health because of their ability to control potentially pathogenic groups, such as *E. coli* (Blomberg *et al*., 1993). Some studies investigating the microbiota of weaned piglets showed that lactobacilli concentrations decreased after weaning (Konstantinov *et al*., 2006b; Mathew *et al*., 1993; Canh and Stutton, 1998). The data presented here showed a similar pattern in lactobacilli 16S rRNA gene copies, which was also consistent with the results obtained by DGGE and sequencing analysis. Moreover, drastic changes of *L. amylovorus* and *L. sobrius* in total *Lactobacillus* populations were also observed in the colon of piglets around weaning, as monitored by *Lactobacillus*-specific PCR-DGGE (Fig. 7. 4). Recently, populations of lactobacilli related to *L. amylovorus* or *L. sobrius* have been identified as common inhabitants of the piglet intestine (Konstantinov *et al*., 2004; 2006b; Janzycyk, 2007), and addition of fermentable carbohydrates can support the growth of these specific lactobacilli in the ileum and colon of weaning piglets (Konstantinov *et al*., 2004).

Generally, periods of intestinal microbiota instability should be the optimal time to feed probiotics to the animal. Due to relatively poor management, piglets from around 10 days of age until after weaning can easily suffer diarrhea in China. It is common practice for most farms in China to provide piglets with creep feed (feed given to young suckling animal in creep) on day 7. Therefore, the present study was designed as such that piglets were orally administrated with strain S1 on days 7, 9 and 11.

Probiotics can beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). It has been assumed that with increasing diversity (i.e.a higher Shannon index), disruption of this balance is more unlikely. Compared with this index of other studies on piglets (Konstantinov *et al*., 2003; 2004), we found in this study that a comparatively complex bacterial community had already developed in hindgut of piglets

on day 7, which may also explain that the effect of strain S1 administration on total bacterial diversity was not significant. On the other hand the comparatively high similarity coefficient between hindgut samples from the same age on day 7, 14, 21 and 24 also confirmed that there were no remarkable changes in the overall microbial community between the two groups. Similar results were also observed in a previous study when fed *L reuteri* MM53 to weaned piglets (Simpson *et al*., 2000). The present study also found that the similarity coefficient between cecal and colonic samples from the same piglet remained high (> 80%) on day 7, 14, 21 and 24, but decreased significantly on day 35. these results indicated that after weaning, unique bacterial community developed gradually in cecum or colon of the individual piglet.

In order to produce the desirable effect, the introduced bacteria must be able to survive gastro-intestinal passage, and be metabolically active in the host's intestine. Several recent studies have indicated that it is common for introduced bacteria to be undetectable 3 to 5 days after termination of treatment (Simpson *et al*., 2000; Walter *et al.*, 2000; Jacobsen *et al*., 1999). In line with these findings, we also demonstrated by *Lactobacillus*-specific PCR-DGGE analysis that populations related to *L. sobrius* had already colonized the colon of piglets before being introduced, but disappeared from the predominant *Lactobacillus* community after weaning (Figure 7. 4). Species-specific real-time PCR assay also showed the same tendency in the numbers of *L. sobrius* copies as the results of DGGE profiles. These results indicated that it should be the optimal time to feed *L. sobrius* to these piglets during weaning transition, although this bacterium appeared on day 35 in some of treatments as shown by the high number of *L. sobrius* in our study. Indeed, a previous study showed that feeding of weaned piglets with *L. sobrius* resulted in a marked increase in abundance of this population in most, albeit not all treated animals (Konstantinov, 2005b).

Our study also found that *Lactobacillus*-specific PCR products obtained from clones most closely related to different species migrated to the identical position in the gel and those corresponding to the same species had different migration positions (Figure 7. 4). This indicated that Shannon index obtained from DGGE profile can not fully reflect the actual diversity of the bacterial community (Nübel *et al*., 1996; Jaspers and Overmann, 2004). To explain this phenomenon, we compared the V2-V3 regions of 16S rRNA gene sequences (*E. coli* positions 159 to 515) of different *Lactobacillus* spp., showing that

several species shared the same sequences (data not shown). Thus, the V2-V3 region might not allow for full resolution of *Lactobacillus* spp. diversity due to limited sequence heterogeneity. To improve this specificity, the intergenic region between 16 and 23S rRNA genes has been investigated for potential design of strain-specific primers (Walter *et al*., 2000; Tannock *et al*., 1999; Haarman and Knol, 2006).

Clostridium spp. are among the dominant bacteria in the hindgut of piglets (Konstantinov *et al*., 2003; Pryde *et al*., 1999). Some species can use glucose to produce butyric acid, which is the preferred energy source for the colonic mucosa and has been implicated in protection against colitis and colorectal cancer (Mclntyre *et al*., 1993; Wachtershauser and Stein, 2000). In the present study, we found that a *C. disporicum* related band only appeared in DGGE profiles from piglets after administration of strain S1. However, to understand the role of this species, both *in vitro* and *in vivo* studies are needed for further investigation.

S. suis is an important cause of a wide variety of infections in pigs, such as meningitis, pneumonia, septicaemia, and arthritis (Clifton-Hadley, 1983; Vecht *et al*., 1992). *S. suis* has also been described as a pathogen for humans (Arends and Zanen, 1988; Trottier *et al*., 1991). In 2005, the pathogen caused a large outbreak of human infection in Sichuan province in China. Of several hundreds of cases, more than 60 were fatal. The present study demonstrated the existence of *S. suis* in the gastrointestinal tract of normal piglets. It seemed that oral administration of strain S1 was able to inhibit the colonization of *S. suis* in the hindgut of piglets after weaning indicating that probiotic treatment can indeed positively affect intestinal microbiota composition, including the competitive exclusion of potential pathogens.

In conclusion, the present study reinforced that weaning can cause a dramatic reduction in the abundance of several microbial populations from the piglet hindgut, most of which belonged to *Lactobacillus* spp. The probiotic strain S1 may have the potential of promoting *C. disporicum* and inhibiting *S. suis*, but further study is still needed to know the relationships between these bacteria and animal health.

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CHAPTER 8

General Discussion

Motivation of this study

Porcine meat is the most important source of animal proteins in China. According to the Ministry of Agriculture of the People's Republic of China, the porcine meat production in 2006 was 51.97 million tons, which is more than 60% of the total meat production of China and 50.1% of global porcine meat production (http://www.info-cma.org). The volume and safety of porcine meat is not only an animal economic issue, but also can affect the social stability in China. The fluctuation of the price of porcine meat relates directly with the quality of life of the Chinese people. Although a full ban on the use of in-feed antibiotics hasn't been in operation so far in China, probiotics and prebiotics as the alternative to antibiotics have attracted increasing interest of animal nutritionists and livestock producers in China. Nevertheless, there is a high variation in the response of the individual animal to probiotics (Simon, 2005). The limited knowledge of diversity and functionality of the normal gut ecosystem provides insufficient clarification of the mechanism of probiotics and prebiotics (Abbott 2004). Nursing is a major critical period in the life of piglets. On one hand maternal antibodies are not able to cross the placenta. Thus, piglets are born without circulating antibodies and consequently lack maternal passive protection (Bauer et al., 2006b). On the other hand, creep-feeding and weaning increase susceptibility to gut disorders, infections and diarrhea (Carstensen *et al.*, 2005; Lallès, et al, 2004). Therefore a better understanding of the composition and function of the normal gut microbiota of piglets is pivotal as a knowledge base for the design of innovative nutritional strategies based on pre-and probiotics to keep piglets healthy.

Aim of this study

The objectives of this study were:

- To describe the composition and function of the intestinal microbiota of piglets during the nursing period through creep feeding and weaning.
- To *in vivo* and *in vitro* evaluate the effect of daidzein on composition and function of intestinal microbiota of nursing piglets in order to evaluate its prebiotic function
- To investigate the probiotic effect of *Lactobacillus sobrius* S1 on composition and function of intestinal microbiota of nursing piglets.

Composition and function of the intestinal microbiota of nursing piglets

For nursing piglets, weaning is a major critical period because of increased susceptibility to gut disorders, infections and diarrhea. It has been reported that intake of a sufficient amount of creep feed during lactation creates a more gradual transition at weaning and can reduce the occurrence of postweaning disorders. However, creep feed consumption during lactation is usually low and is also highly variable among piglets within and between litters (Barnett et al., 1989; Pajor et al., 1991; Kuller et al., 2007). It is now well accepted that the animal host largely benefits by carrying a diverse and dense microbiota. This includes an improved development of intestinal epithelium, lymphoid tissue, contributions to host nutrition, and the colonization resistance to pathogens (Hooper and Gordon, 2001; Backhed et al., 2005). The structure and composition of the gut microbiota reflects natural selection at two levels: at the microbial level, where lifestyle strategies (e.g., growth rate and substrate utilization patterns) affect the fitness of individual bacteria in a competitive ensemble; and at the host level, where suboptimal functionality of the microbiota can reduce host fitness (Backhed et al., 2005). The nursing period is a critical period for the establishment of the gastrointestinal microbiota of piglets. On one hand the microbiota is yet un-established. On the other hand, the host piglets are rapidly growing, and their diet shifts from maternal milk to solid formula. At the microbial level, to benefit the host, bacteria from mother, diet and environment must be organized in a stable food web that aids in breaking down nutrients, provides the host with energetic substrates, and aid in feeding each other to form a fairly stable community. At the host level, multiple levels of selection occur according to host development of physiological function and immune system associated with its rapid growth and diet shifting. Early creep-feeding increases the diversity of the microbiota and stabilizes the microbiota of piglets around the weaning period (chapter 3). High diversity is generally thought to be desirable for ecosystem stability (McCann, 2000). One important mechanism by which diversity can confer resilience is through providing of a wide repertoire of responses to stress (Yachi and Loreau, 1999). That implies that creep feeding is beneficial to reduce the pressure of weaning on the intestinal microbiota of piglets. On the other hand, experiments described in this thesis also provided evidence that early creep-feeding can increase the instability of microbiota of suckling piglets (chapter 3). Some diet components of creep feed, such as

soy protein, may act as feed antigens and can induce an immune reaction in creep-fed pigs (Barnett *et al.* 1989). The immune system is the host's first line of contact with the microbiota in the gut and can be expected to play a role in shaping the microbiota (Ley *et al*., 2006).

Lactobacilli are important members of a healthy, balanced microbial community in the GI-tract. It is considered that they directly affect the host's health. It has been postulated that *Lactobacillus* spp. has several health-promoting effects. These include 1) immuno-stimulation by association with intestinal mucosa, 2) alleviation of food intolerance and allergy by production of hydrolytic enzymes, 3) prevention of diarrhea and intestinal infections by production of lactic acid, acetic acid and antimicrobials (Servin, 2004; Bengmark *et al*., 2000; Ouwehand *et al*., 1999; Salminen *et al*., 1998).

Lactobacilli are among the early colonizers of the porcine intestine and go through a succession over time associated with host growth and the drastic shifts in diet (chapter 4). Within the *Lactobacillus* community, some members like *L. reuteri* and *L. amylovorus/sobrius* function as entrenched residents, while others such as *L. delbrueckii*, *L. acidophilus* and *L. crispatus* can be regarded more as allochthonous and transient members derived from ingested feed, water, and the environment (chapter 4; Konstantinov et al., 2006). Creep feed can change the pattern of diversity fluctuation of the *Lactobacillus* community. For piglets with creep feeding, the most obvious fluctuation of diversity could be observed between creep feeding and weaning (chapter 4), whereas for piglets without creep feeding, it happened within one week after weaning (chapter 7, Fig 7. 4).

Daidzein as a potential prebiotic to support porcine growth and health

A prebiotic has been defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health (Gibson, 2004). In order for a food ingredient to be classified as a prebiotic, it must 1) be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract; 2) be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated; 3) consequently, be able to alter the colonic microbiota

in favor of a healthier composition; and 4) induce systemic effects that are beneficial to the host health (Gibson, 1995).

Soybeans and soy products are the most significant dietary sources of daidzein and its glycoside daidzin (Coward *et al*., 1993; Wang *et al*., 1994). All soybean proteins and foods currently available for human and animal contain significant amounts of daidzein and/or daidzin. Daidzein and other isoflavones are phytoestrogens, and their bioactivity in humans and animals has been well reviewed (Kurzer and Xu, 1997; Murkies *et al*., 1998; Setchell, 1998; Han *et al*., 2006). Setchell et al. (2002a) clearly showed that isoflavone glucosides (daidzin) are not absorbed intact across the enterocytes of healthy adults. Its bioavailability requires initial hydrolysis of the sugar moiety by intestinal β-glycosidase activity before uptake into the peripheral circulation. It is generally accepted that this does not occur until the compounds reach the colonic microbiota, which produce β-glycosidase (King and Bursill 1998). Day et al. (2000) found that lactase phlorizin hydrolase, a β-glycosidase present in the brush border of the mammalian small intestine, is capable of hydrolyzing isoflavone glycosides. Nevertheless, a substantial amount of isoflavone glycosides degradation in the large intestine can be expected because of the limited residence time of food compounds in the small intestine and a possible decrease of lactase phlorizin hydrolase levels into adulthood (Harvey et al. 1995). Its aglycone (daidzein) can further undergo the fermentation by human and animal intestinal bacteria. It has been convincingly shown that colonic microbiota play an important role in the metabolism of daidzin and daidzein. The hydrolyzation of isoflavone glycosides can stimulate their absorption, and their further conversion (especial equol-production) can enhance their estrogenic potency *in vivo* (review by Setchell *et al*., 2002b). Up to now eight strains involved in the conversion of daidzin and daidzein have been isolated from human feces, and single isolates have been obtained from rat caecum and the rumen, respectively.

Besides the degradation of daidzin and daidzein by gut microbiota, the multiplication of some bacterial species promoted by daidzin and daidzein has been also observed clearly. De Boever *et al*. (2000) studied the effect of a soygerm powder rich in β-glycosidic isoflavones (daidzin and genistin) on the fermentation pattern of the colon microbiota of healthy humans by using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The supplement of 2.5 g/d of soygerm powder which provided 62.5 mg/d of isoflavone promoted an overall increase of populations of *Enterobacteriaceae*, coliforms,

Lactobacillus spp., *Staphylococcus* spp. and *Clostridium* spp., with a significant increase of the *Lactobacillus* spp. population. The short-chain fatty acid (SCFA) concentration increased with 30% during the supplementation period, which was due mainly to a significant increase of acetic and propionic acids. This is in line with the results of our *in vitro* evaluation (chapter 5), which showed that 50 mg/L of daidzein does not affect *Lactobacillus* community composition in ileal and colonic chyme of piglets in batch incubations, but it significantly increased overall relative abundance of lactobacilli. The proportion of propionate in TVFA by all inocula, and lactate production by foregut inocula were increased remarkably by daidzein. These two *in vitro* studies have provided direct evidence that daidzein and its glycoside can exert selective growth promoting effects on members of the human and porcine intestinal microbiota, especially lactobacilli (Fig. 8.1). Furthermore, research described in chapter 6 showed an *in vivo* effect of daidzein on the colonic microbiota composition of piglets. One mg oral supplement of daidzein to suckling piglets at 7, 9 and 11 days of life induced the appearance of three populations that were most closely related to *Clostridium butyricum*, a yet uncharacterized butyrate-producing bacterium within the clostridia, and *Ruminococcus obeum*, respectively, and the disappearance of some bacteria especially within the genus *Streptococcus* after weaning.

Furthermore, daidzein can increase the survival of *Lactobacillus* spp. in bile salt. De Boever *et al*. (2001) investigated the survival of *Lactobacillus reuteri* when challenged with glycodeoxycholic acid (GDCA), deoxycholic acid (DCA) and soygerm powder. When 4 g /L soygerm powder (including 44.9± 8.4μmol/L and 12.4 ±2.9 μmol/L for daidzein and genistein, respectively) was added, the *Lactobacillus* strain survived the bile salt burden better ($P < 0.05$) and the membrane damage in the haemolysis test decreased ($P < 0.05$). Su *et al*. (2005) confirmed this observation using the piglet gut isolate *Lactobacillus sobrius* S1. With 50 mg/ L supplement of daidzein, the S1 survival counts increased significantly under bile salt conditions. The antioxidant property of daidzein should be the reason that it can decrease the damaging effect that bile salt have on lactobacilli. Zavodnik (2003) reported that rat treatment with genistein-8-C-glicoside (75 mg/kg) had a clear protective effect, stabilized membrane structure and improved the parameters of the monooxygenase function.

In conclusion, daidzein has prebiotic potential towards improved porcine growth and

health, however, the mechanisms of the beneficial effects of daidzein are various comparing to commonly used prebiotics, such as oligosaccharides.

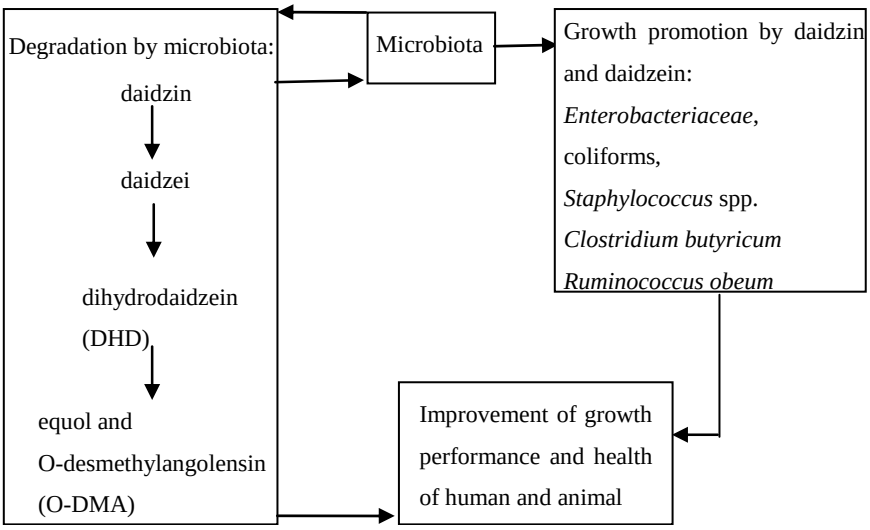

Fig 8.1. the possible relationship between intestinal microbiota, daidzein and host health.

Lactobacillus sobrius S1: a member of the porcine resident microbiota and its probiotic potential

A recent formal definition of probiotics was agreed upon by a working group of European scientists and given as a live microbial feed supplement that is beneficial to health (Salminen and others 1998). The following criteria must be met before a probiotic can be described as useful (Fuller 1991 and 1992): 1) The probiotic must be capable of being prepared in a viable manner and on a large scale (e.g., for industrial purposes). 2) During use, and under storage, the probiotic should remain viable and stable. 3) It should be able to survive in the intestinal ecosystem. 4) The host animal should benefit from harboring the probiotic.

Within a given intestinal habitat, some microbial members function as entrenched "residents" (autochthonous components), while others act more like hitchhikers (allochthonous members) from ingested food, water, and various components of our environment (Ley *et al*., 2006). This has also recently been shown for the human gut microbiota, where high throughput analysis of fecal samples of healthy volunteers taken over a period of 10 years using a comprehensive DNA microarray platform, the Human Intestinal Tract Chip (HITChip), has provided evidence for a subject-specific core microbiota stable in time (Rajilić-Stojanović, 2007). These core residents can be inherited

vertically from the mother, can maintain and restore themselves through self-replication, and can be the mutualists to the host. Consequently the healthy gut ecosystem is in a dynamic stable situation (Backhed *et al*., 2005; Ley *et al*., 2006). This may be the reason why the probiotics are likely to function when the host is in some sort of stressed state, such as weaning and diarrhea. Furthermore, a probiotic strain derived from the resident members of the intestinal microbiota of a specific host may be able to survive in the intestinal ecosystem easily. In contrast, Bouhnik et al. (1992) indicated that a *Bifidobacterium sp*. ingested from fermented milk was rapidly washed out of the colon when it was no longer consumed.

Lactobacillus sobrius S1 is an *L. amylovorus*-like isolate derived from piglet intestine (chapter 4), which is stably present in the colon. It is closely related to the type strain, *L. sobrius* OTU171-001, previously isolated from the intestine of piglets (Konstantinov *et al*., 2006). S1 can rapidly produce lactate and decrease the pH value of medium. Consequently, it could also be shown to inhibit growth of *E. coli* K88, K99, 987P and O141. The production of lactate should be the main reason of that effect, because heat treatment and protease treatment only slightly decreased the inhibition, whereas it was reduced by 47% to 78% after excluding the effect of lactate (Wu et al., 2005a). S1can survive in medium with pH 1.5 for 30 min, and can survive in medium with a low concentration of bile salts below 0.2%. S1 was also shown to tolerate heat treatment for 5-15 minutes at temperatures below 80°C (Wu et al., 2005b). These results indicate that S1 can be industrially processed during feed manufacturing, and can remain viable and stable during storage. During use, it can survive the presence of gastric acid and bile salt, and thus survive in the intestinal ecosystem.

The probiotic function of a S1 preparation was evaluated by a limited number of animal trials. 32 litters of newborn piglets were divided into four groups, control, and three S1 treatment groups that received 10^7 CFU·ml^{-1}, 10^9 CFU·ml^{-1} or 10^{11} CFU·ml^{-1} of the S1 preparation, respectively, through oral administration at 7, 9 and 11 days of age. Results showed that all S1 oral administrations significantly decreased the occurrence of piglet diarrhea, with the most remarkable effectiveness of 10^9 CFU·ml^{-1} administration, namely a reduction to 4.4% from 9.1% of the diarrhea levels observed in the control group (unpublished data). Such dose-dependent differences in efficacy of probiotic treatment have also been recently described for treatment of women suffering from irritable bowel

syndrome with a bifidobacterial preparation (Whorwell et al., 2006). The other trial used 120 litters of piglets from three different farms to evaluate the effect of an S1 preparation on growth performance and the occurrence of diarrhea through 10^9 CFU·ml^{-1}of S1 oral administration at 7, 9 and 11 days of age. Results showed that the average body weight gain of the treatment group increased 9.5% comparing to the control group. This is in line with previous observations from a trial, where the closely related probiotic strain *L. sobrius* OTU171-001 was given to weaned piglets challenged with ETEC (Konstantinov, 2005). The occurrence of diarrhea in the treatment group was apparently less than control group, 3.1% and 19.7% respectively (unpublished data). These results showed that the host piglets gain benefits from oral administration of S1 preparation.

The underlying mechanism has been partly investigated in studies described in chapter 7. Weaning can cause a dramatic reduction in the abundance of several microbial populations from the piglet hindgut, most of which belonged to *Lactobacillus* spp. S1 had the potential of promoting beneficial bacteria and inhibiting potential pathogens. S1 could also increase the VFA concentration in the colon of piglets, with an increase of butyrate concentration (Su *et al*., 2006). The improvement of gastrointestinal development has been observed in the same piglet trial as well (Lu et al., 2006). S1 could increase the number of oxyntic cells in the fundic gland of piglets after weaning ($P < 0.01$) and could increase the height of duodenal villi.

Thus, oral administration of *Lactobacillus sobrius* S1 can positively affect host piglet health and its intestinal microbiota. S1 is a potential probiotic strain for porcine growth and health, reinforcing previous findings obtained using *L. sobrius* OTU171-001 as a probiotic in weaning piglets challenged with *E.coli K88* (Konstantinov, 2005).

Conclusion and perspective

This thesis leads to several general conclusions: 1) Early creep-feeding stabilizes the microbiota of piglets around the weaning period. 2) *Lactobacillus* communities follow a successional change associated with piglet growth and diet shifting. Creep feeding stabilizes the *Lactobacillus* community of weaning piglets. Within the *Lactobacillus* community, some members like *L.reuteri* and *L.amylovorus/L.sobrius* might be permanent colonizers, while *L.delbrueckii, L.acidophilus* and *L.crispatus* might be transient members of the *Lactobacillus* communities in the piglet´s GI tract. 3) Both *in vitro* and *in vivo*

evaluations indicated that daidzein has the potential for use as a prebiotic substance in animal feed. 4) *Lactobacillus sobrius* S1 has the potential of promoting beneficial bacteria and inhibiting potential pathogens.

The prebiotic effect of daidzein and the probiotic effect of *Lactobacillus sobrius* S1 have been observed in this thesis, and the protection of S1 by daidzein has been reported (De Boever *et al*. 2001; Su *et al*., 2005). The combined use of daidzein and *Lactobacillus sobrius* S1 will be of interest for future research. To clarify the function of the intestinal microbiota, the integration of research areas and the combination of cultivation-dependent and molecular methods, including functional genomics approaches, will require additional attention.

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Summary

Although a full ban on the use of in-feed antibiotics hasn't been in operation so far in China, probiotics and prebiotics as the alternative to antibiotics has attracted increasing interest of animal nutritionists and livestock producers in China. Nevertheless, there is a high variation in the response of the individual animals to probiotics (Simon, 2005). The limited knowledge of diversity and functionality of the normal gut ecosystem provides insufficient clarification of the mechanism of probiotics and prebiotics (Abbott 2004). Nursing is a major critical period in the life of piglets. On one hand maternal antibodies are not able to cross the placenta. Thus, piglets are born without circulating antibodies and consequently lack maternal passive protection (Bauer et al., 2006b). On the other hand, creep-feeding and weaning increase susceptibility to gut disorders, infections and diarrhea (Carstensen *et al.*, 2005; Lallès, et al, 2004). Therefore clarification of the composition and function of the normal gut microbiota of piglets is pivotal as a knowledge base for the design of innovative nutritional strategies based on pre-and probiotics to keep piglets healthy.

The objectives of this study were to describe the composition and function of the intestinal microbiota of piglets during the nursing period during creep feeding and weaning, to *in vivo* and *in vitro* evaluate the effect of daidzein on composition and function of intestinal microbiota of nursing piglets in order to evaluate its prebiotic function, and to investigate the probiotic effect of *Lactobacillus sobrius* S1 on composition and function of intestinal microbiota of nursing piglets.

Isoflavones are a certain class of estrogenic compounds that are often associated with a reduced risk of cancers. Their estrogenic activity can be enhanced after metabolization into more active compounds such as equol by gut microorganisms. The direct use of these metabolites has been investigated in laboratory rats and farm animals over the last decade. **Chapter 2** reviews the research progress on metabolism of isoflavones in the intestinal tract. This includes the role of intestinal microbiota in its metabolism, isolation of isoflavone-degrading bacteria and the relationship between equol-producing capacity and the composition of individual microbiota, and the research progress with respect to the effect of isoflavonic compounds including metabolites on the physiology, gut microbiology and performance of farm animals in China.

Chapter 3 describes the monitoring of the development and diversity of the fecal bacterial community of 5 newborn diarrhea piglets, which were observed with yellow soft feces at 2 days of age using a 16S ribosomal RNA (rRNA) approach. DGGE profiles of 5 piglets changed from simple (day 2) to complex (day 10), and then from simple (day 16) to complex (at weaning, day 28) again, and finally remained relatively stable and diverse after weaning. DGGE profiles from fecal samples taken on day 2 and 16 were highly simple and similar, and dominated by a single band corresponding to *E. coli*. DGGE profiles from day 10 fecal samples were more complex, and the band corresponding to *E. coli* was still present, albeit not predominantly. DGGE profiles from day 35 and 42 fecal samples became complex again, while their predominant bands remained similar and stable. 16S rRNA gene sequence analysis revealed that the 23 clones from the library generated from fecal samples of day 42 health piglets were most closely related to species of *Enterococcus*, *Streptococcus*, *Clostridium*, *Peptostreptococcus*, *Lactobacillus* and *Bacillus*.

Molecular diversity and development of the *Lactobacillus* community in the intestinal tract of conventionally raised piglets, as influenced by age, diet and gut compartment were studied in **chapter 4**. DGGE profiles revealed that the *Lactobacillus*-like communities throughout the GI tract from duodenum to rectum showed good stability at same age. Two dominant bands were found for microbiota in the tissue of the small intestine. This indicates that the lactobacilli in chyme can adhere to the small intestinal wall. The *Lactobacillus* communities in different GI tract compartments developed over time. A successional change of *Lactobacillus* communities was observed from birth, through creep feeding to one week after weaning, with a trend from simple to complex and again to simple. Furthermore, a clone library of *Lactobacillus*-specific PCR products was generated from jejunal and colonic digesta. The clones corresponding to dominant DGGE bands were matched with sequences derived from *L.reuteri*, *L.delbrueckii*, *L.crispatus*, *L.amylovorus/L.sobrius*, and *L.acidophilus*. Amplicons related to *L.reuteri* were found in all DGGE fingerprints from jejunal digesta of week 1, 3 and 4. Amplicons related to *L.amylovorus* and *L.sobrius* were detected in all DGGE fingerprints from colonic digesta of piglets of 1, 3 and 4 weeks of age. Amplicons related to *L.delbrueckii* were found before weaning, *L.crispatus* after creep feeding before weaning, and *L.acidophilus* after

weaning. This indicates that *L.reuteri* and *L.amylovorus / L.sobrius* probably belong to the permanent colonizers, whereas *L.delbruckii, L.acidophilus* and *L.crispatus* more likely are transient inhabitants of the piglet GI tract.

In vitro investigation of the impact of the phytoestrogen daidzein on compositional and functional aspects of the porcine ileal and colonic *Lactobacillus* community during the nursing period was conducted in **chapter 5**. Ileal and colonic chymes from one litter of 12 conventionally raised piglets, which were euthanized at 1, 2, 3 (weaning) and 4 weeks of age (3 animals each), were used as inocula in *in vitro* treatments, respectively: Van Soest medium with (a) 0.5g of glucose and 5mg of daidzein, (b) 0.5g of glucose, and (c) Van Soest medium only. After 48 h of fermentation, the ileal microbiota produced more gas, more total volatile fatty acid (TVFA) and more lactic acid than the colon microbiota on week 1. On week 4, more gas and TVFA were produced in the colonic fermentation. Daidzein did not affect DGGE patterns of the *Lactobacillus* community. Dilution PCR, however, indicated that daidzein increased the relative abundance of *Lactobacillus* irrespective of intestinal compartment and piglet age. This was confirmed by MRS plate counts and lactic acid production, and the results suggest that daidzein may have the potential for use as a prebiotic substance in animal feed.

The *in vivo* effect of daidzein on the intestinal bacterial communities of piglets was studied by 16S rRNA-based techniques in **chapter 6**. Within 3 litters, piglets were randomly allocated to two groups: control and treatment group. The piglets of the treatment group were fed 1 mg of daidzein as pure extract orally on day 7, 9 and 11. All piglets were weaned on day 21. One piglet from every litter of each group was randomly slaughtered on day 14, 21, 24 and 35, respectively. Piglets of the treatment group showed a higher bacterial diversity and more DGGE bands compared with those of the control group on days 24 and 35. Three DGGE bands observed in the control were absent in the treatment group profiles. Cloning and sequence analysis revealed that the corresponding populations were mostly closely related to *Clostridium thermocellum*, *Lactobacillus pontis* and *Streptococcus* sp., respectively. Three other DGGE bands were present only in the treatment group, and their corresponding sequences exhibited highest similarity to that of Butyrate-producing bacterium SL7/1, *Clostridium butyricum*, and *Ruminococcus obeum*, respectively.

Chapter 7 described changes in the composition of microbiota in the hindgut of piglets after oral administration of *Lactobacillus sobrius* S1, using molecular techniques based on 16S rRNA genes. Six litters of neonatal piglets were divided randomly into a control group and a treatment group. At 7, 9, and 11 days of age, piglets in the treatment group orally received a preparation of *L. sobrius* S1. At 7, 14, 21(weaning), 24 and 35 days of age, one piglet from each litter was sacrificed and digesta samples from the hindgut were collected. DGGE analysis for all bacteria showed that several populations present in the hindgut of piglets, represented by far-migrating bands, disappeared after weaning. Sequence analysis indicated that most of these bands corresponded to *Lactobacillus* spp. This trend was also confirmed by quantitative real-time PCR. Drastic changes of *L. amylovorus* and *L. sobrius* in total *Lactobacillus* populations were also observed in the colon of piglets around weaning, as monitored by *Lactobacillus*-specific PCR-DGGE analysis. Comparison of DGGE profiles between control and treatment groups revealed a specific band related to *Clostridium disporicum* that was found only in the treatment group on day 14. On day 35, a specific band appeared only in the control group, representing a population most closely related to the potential porcine pathogen *Streptococcus suis* (99%). Species-specific real-time PCR revealed that the population of *L. sobrius* declined apparently in the colon of piglets after weaning, while it tended to re-establish earlier after oral administration of strain S1.

Finally the main achievements of the current study are discussed. This thesis leads to several general conclusions: 1) Early creep-feeding stabilizes the microbiota of piglets around the weaning period. 2) *Lactobacillus* communities follow a successional change associated with piglet growth and diet shifting. Creep feeding stabilizes the *Lactobacillus* community of weaning piglets. Within the *Lactobacillus* community, some members like *L.reuteri* and *L.amylovorus/L.sobrius* might be permanent colonizers, while *L.delbruckii, L.acidophilus* and *L.crispatus* are more likely to be transient members of the *Lactobacillus* communities in the piglet´s GI tract. 3) Both *in vitro* and *in vivo* evaluations indicated that daidzein has the potential for use as a prebiotic additive in animal feed. 4) *Lactobacillus sobrius* S1 has the potential of promoting beneficial bacteria and inhibiting pathogens.

Samenvatting

In tegenstelling tot de Europese Unie bestaat er in China nog geen volledig verbod op het gebruik van antibiotica als groeibevorderaars. Net als elders wordt gezocht naar alternatieven,.Men onderzoekt speciaal de mogelijkheden van probiotica en prebiotica als alternatieven voor dit soort antibiotica. Het krijgt steeds meer aandacht van voedingsdeskundigen en veehouders in China. Er is een zeer grote variatie in reacties van individuele dieren op probiotica (Simon, 2005). Onze kennis van de diversiteit en functies van het normale darm ecosysteem is nog beperkt en we hebben geen eenduidige verklaring voor het werkingsmechanisme van probiotica and prebiotica (Abbott 2004).

De zoogperiode is een belangrijke en kritische fase in het leven van biggen. Aan de ene kant kunnen maternale antilichamen de placenta niet passeren. De biggen worden dus geboren zonder circulerende antilichamen en zij missen de passieve bescherming `door deze antilichamen (Bauer et al.,2006b). Aan de andere kant kunnen bijvoeding en spenen de gevoeligheid voor darmstoornissen, infecties en diarree doen toenemen (Carstensen *et al.*, 2005; Lallès, et al, 2004). Daarom is kennis over de samenstelling en functie van de normale darm microflora van essentieel belang om innovatieve voedingsstrategieën te kunnen ontwerpen op basis van pre- en probiotica met als doel de biggen gezond te houden rond de speenperiode.

De doelstelling van dit onderzoek was het omschrijven van enkele aspecten van samenstelling en functie van darm microflora bij biggen gedurende de hele zoogperiode en gedurende het deel van de zoogperiode waarin biggen bijvoeding krijgen. De studie omvatte ook de periode rond het spenen. Middels *in vivo* and *in vitro* methoden werd het effect van daidzein (een isoflavoon uit soja) nagegaan op samenstelling en functie van darm microflora van biggen gedurende de zoogperiode. Doel was de mogelijke prebiotische functies van daidzein te onderzoeken. Daarnaast werd een mogelijk probotisch effect van *Lactobacillus sobrius* S1 op samenstelling en functie van darm microbiota van biggen gedurende deze levensperiode bestudeerd.

Isoflavonen behoren tot een groep van oestrogene componenten die geassocieerd worden met verminderd risico voor bepaalde typen kanker. De oestrogene activiteit van Dadzein kan nog verhoogd worden nadat de darm microflora het daidzein omgezet heeft in componenten die meer actief zijn zoals equol. Het gebruik van deze metabolieten in plaats van daidzein zelf is gedurende de laatste 10 jaar onderzocht bij laboratorium ratten en bij landbouwhuisdieren.

Hoofdstuk 2 geeft middels een literatuurstudie een overzicht van de voortgang in het onderzoek naar het metabolisme van isoflavonen uit soja in het maagdarmkanaal. Het beschrijft aspecten van (1)de rol van micro-organismen voor dit metabolisme (2) ,de isolatie van isoflavoon-afbrekende bacterien in het maagdarmkanaal (3) de relatie tussen het vermogen om equol te produceren uit daidzein en (4) de relatie van deze 3 met de samenstelling van de microflora. Er wordt een overzicht gegeven van de vooruitgang in het onderzoek met betrekking tot het effect van isoflavone componenten en hun metabolieten op dieren. Hierbij wordt gekeken naar fysiologische aspecten naar darmmicrobiologie en naar productie van landbouwhuisdieren in China.

In Hoofdstuk 3 wordt beschreven hoe de ontwikkeling van de darm microflora en de diversiteit in fecale microflora verloopt in 5 biggen die vanaf een paar dagen na de geboorte gevolgd werden. Deze 5 biggen werden gekozen omdat ze geboorte diarree hadden. Deze diarree wordt gekarakteriseerd door gele zachte mest op een leeftijd van twee dagen. De microflora werd bestudeerd met gebruikmaking van de 16S ribosomale RNA (rRNA) benadering. DGGE profielen van bacteriën uit de mest van deze 5 biggen veranderde van vrij eenvoudig (op dag 2) tot complex (op dag 10) en vervolgens weer van vrij eenvoudig (op dag 16) tot complex bij spenen (op dag 28). Na het spenen bleef de darm microflora relatief stabiel en divers. DGGE profielen van mest monsters genomen op dag 2 en dag 16 waren niet erg divers en leken op elkaar. Het profiel werd gedomineerd door een enkele band die correspondeerde met de aanwezigheid van *E. coli*. DGGE profielen in mestmonsters vanaf dag 10 waren veel meer complex. De band die correspondeert met *E. coli* was bij de oudere dieren nog wel aanwezig maar niet meer zo dominant. DGGE profielen in de mest van deze dieren van dag 35 en dag 42 werden veel complexer met dezelfde dominante en stabiele banden. 16S rRNA gen sequentie analyse liet zien dat de 23 klonen van de bibliotheek die gegenereerd werden uit mest monsters van gezonde biggen van dag 42 het meest overeen kwamen met soorten van *Enterococcus*, *Streptococcus*, *Clostridium*, *Peptostreptococcus*, *Lactobacillus* en *Bacillus*.

Moleculaire diversiteit en ontwikkeling van de *Lactobacillus* gemeenschap in het maagdarmkanaal van biggen die op conventionele manier worden gehouden is beschreven in **hoofdstuk 4**. DGGE profielen gaven aan dat de *Lactobacillus*-achtige microbiële gemeenschappen van duodenum tot rectum een goede stabiliteit hadden bij dezelfde

leeftijd. Twee dominante banden voor lactobacilli werden gevonden in monsters van dunne darmweefsel. Dit laat zien dat de lactobacilli in de chymus ook aanwezig kunnen zijn aan de wand van de dunne darm.. De *Lactobacillus* gemeenschappen in de verschillende compartimenten van het maagdarm kanaal ontwikkelen zich in de tijd met het veranderen van de leeftijd van de big. Er werd een soort opvolgingsverandering waargenomen in *Lactobacillus* gemeenschappen vanaf geboorte tot bijvoeding tijdens de zoogperiode en later tot een week na spenen. De trend was van eenvoudig tot complex en later opnieuw naar eenvoudig. Bovendien werd een kloon bibliotheek van *Lactobacillus*-specifieke PCR producten gegenereerd uit chymus van jejunum en colon. De klonen corresponderen met dominante DGGE banden en sequentie analyse wees uit dat ze afkomstig waren van *L.reuteri, L.delbrueckii, L.crispatus, L.amylovorus/L.sobrius,* en *L.acidophilus*. De amplicons die gerelateerd waren met *L.reuteri* werden waargenomen in alle DGGE fingerprints uit chymus van jejunum van biggen die 1, 3 en 4 weken oud waren. Amplicons die gerelateerd waren aan *L.amylovorus* en *L.sobrius* werden ontdekt in alle DGGE fingerprints uit chymus van colon afkomstig van al deze biggen. Amplicons gerelateerd aan *L.delbrueckii* werden gevonden in chymus van biggen voor het spenen. Verder werd *L.crispatus* gevonden na bijvoeren tijdens de zoogperiode en *L.acidophilus* na het spenen. Dit geeft aan dat *L.reuteri* en *L.amylovorus / L.sobrius* waarschijnlijk behoren tot de permanente kolonisatoren, terwijl *L.delbruckii, L.acidophilus* en *L.crispatus* waarschijnlijk meer de tijdelijke bewoners van het maagdarmkanaal van biggen zijn.

In vitro onderzoek naar de impact van het phytoestrogeen daidzein op aspecten van samenstelling en functie van de *Lactobacillus* gemeenschap in ileum en colon gedurende de zoogperiode is beschreven in **hoofdstuk 5**. Chymus uit ileum en colon van 12 conventioneel gehouden biggen van een toom werden gebruikt als inocula. Steeds werden3 biggen geeuthaniseerd op een leeftijd van 1 week, 2 weken, 3 weken (bij spenen) en 4 weken. De chymus inocula werden voor drie verschillende *in vitro* behandelingen gebruikt: Van Soest medium met (a) 0.5g glucose en 5mg daidzein, (b) 0.5g glucose, en (c) alleen Van Soest medium. Na 48 uur fermentatie produceerde de ileale microflora uit biggen van 1 week oud meer gas, meer vluchtige vetzuren (TVFA) en meer melkzuur dan de colon microflora van dezelfde biggen. Echter, Inocula uit de colon van biggen van 4

weken oud produceerde meer gas en meer TVFA dan inocula uit het ileum. Daidzein had geen effect op DGGE patronen van de *Lactobacillus* gemeenschap. Analyse met Dilution PCR, liet echter zien dat daidzein zorgde voor relatief hogere aantallen van *Lactobacillus* na incubatie met inoculum uit alle darmdelen en onafhankelijk van de leeftijd van de biggen. Dit werd bevestigd door MRS plaattellingen en melkzuur productie cijfers. De resultaten suggereren dat daidzein de potentie heeft om als prebioticum in diervoeder te worden gebruikt .

Het *in vivo* effect van daidzein op de microflora in de darm van biggen werd bestudeerd met kweek-onafhankelijke 16S rRNA-gebaseerde technieken. Dit onderzoek is beschreven in **hoofdstuk 6.**. In drie tomen werden de biggen ad random toegewezen aan een van twee behandelingen: controle of behandeling. De biggen van de behandelingsgroep kregen oraal 1 mg daidzein als zuiver extract in ondermelk telkens op dag 7, 9 en 11. De controle dieren kregen alleen ondermelk. Alle biggen werden gespeend op dag 21. Telkens werd een big van elke toom van elke behandeling ad random gekozen en geeuthaniseerd op respectievelijk dag 14, 21, 24 en 35. Biggen die behandeld waren met daidzein toonden op dag 24 en ook op dag 35 een grotere diversiteit in bacteriele microflore. Er werden meer DGGE banden bij deze dieren waargenomen in vergelijking met de controle dieren. Drie DGGE banden die waargenomen werden bij controle dieren waren niet te zien in profielen van de met daidzein behandelde dieren. Kloneren en sequentie analyse lieten zien dat de corresponderende populaties het meest gerelateerd waren aan *Clostridium thermocellum*, *Lactobacillus pontis* en *Streptococcus* sp.. Drie andere banden werden alleen gezien in de behandelde dieren en correspondeerden met sequenties die grote overeenkomst hebben met respectievelijk die van butyraat-producerend bacterium SL7/1, *Clostridium butyricum*, en *Ruminococcus obeum*.

Hoofdstuk 7 beschrijft de verandering in samenstelling van de microbiota in de einddarm van biggen na orale toediening van *Lactobacillus sobrius* S1, Hierbij werden moleculaire technieken gebaseerd op 16S rRNA genen gebruikt. Zes tomen van pasgeboren biggen werden gebruikt. Biggen van elke toom warden ad random ofwel aan de controle groep ofwel aan de behandelingsgroep toegewezen. Op een leeftijd van 7, 9, en11 dagen kregen de biggen van de behandelings groep oraal een preparaat met *L. sobrius* S1 en de controle dieren kregen alleen de drager. Telkens werden op dag 7, 14, 21 (bij het spenen)

en op dag 24 en 35 een big van elke behandeling van elke toom geeuthaniseerd en chymus monsters verzameld uit het einde van de dikke darm. DGGE analyse voor alle bacterien liet zien dat meerdere populaties die aanwezig zijn in het laatste deel van het maagdarm kanaal van biggen verdwenen na het spenen. Sequentie analyse toonde aan dat de meesten van deze banden, welke gevonden werden in het onderste gedeelte van de DGGE , correspondeerden met *Lactobacillus* spp. Deze trend werd ook bevestigd met behulp van quantitatieve real-time PCR techniek. Er werden zeer grote veranderingen in populaties van *L. amylovorus* / *L. sobrius* en in totale *Lactobacillus* waargenomen in het colon van biggen rond het spenen. Dat werd geconcludeerd uit *Lactobacillus*-specific PCR-DGGE analyse. Vergelijking van DGGE profielen van microbiota uit controle- en behandelingsdieren laat zien dat de specifieke band gerelateerd aan *Clostridium disporicum* alleen werd gevonden in dieren uit de`behandelingsgroep van 14 dagen oud. Een andere specifieke band verscheen alleen in chymus van controle dieren van 35 dagen. Deze is representatief voor een populatie die het meest gerelateerd is aan de potentieel pathogene *Streptococcus suis* (99% sequentie overeenkomst). Specifieke kwantitatieve PCR liet zien dat de populatie van *L. sobrius* na het spenen van de biggen sterk verminderd was in het colon, maar sneller herstelde in dieren die behandeld waren met stam S1.

In hoofstuk 8 worden de meest belangrijke bevindingen besproken van de studies zoals die beschreven zijn in dit proefschrift.. De meest belangrijke conclusies zijn: 1) Vroeg bijvoeren draagt bij aan een stabilisatie van de microflora van biggen rond het spenen. 2) De *Lactobacillus* gemeenschap is onderworpen aan veranderingen in de tijd, die samenhangen met toenemende groei van dieren en veranderingen in de voeding. Bijvoeding stabiliseert de *Lactobacillus* gemeenschap rond het spenen. Enkele populaties zoals *L.reuteri* en *L.amylovorus/L.sobrius* zijn waarschijnlijk permanente koloniseerders, daarentegen komen *L.delbruckii, L.acidophilus* en *L.crispatus* waarschijnlijk alleen gedurende korte tijd voor in de darm van de biggen. 3) Zowel *in vitro* als ook *in vivo* metingen hebben uitgewezen dat daidzein potentie heeft als prebiotisch additief in diervoeding. 4) *Lactobacillus sobrius* S1 kan mogelijkerwijs de goedaardige microflora bevorderen en potentieel pathogene bacteriën remmen.

摘 要

尽管中国目前还没有在动物饲料中全面禁用抗生素，但是益生素和化学益生素这两种抗生素替代品已经引起了畜牧生产者和动物营养学者的高度兴趣和重视。然而，益生素在应用时，由动物个体差异带来的效果差异非常明显(Simon, 2005). 对动物肠道正常微生物区系了解的局限是对益生素和化学益生素作用机制缺乏充分阐述的根本原因(Abbott 2004). 保育期是仔猪生产中的一个重要阶段。一方面，母源抗体无法通过胎盘,因此仔猪出生时不能获得母源抗体的保护(Bauer et al., 2006b)。另一方面，开食和断奶又会增加仔猪腹泻、肠道感染等的可能(Carstensen *et al.*, 2005; Lallès, et al, 2004)。因此，澄清仔猪肠道正常微生物区系的结构和功能有助于准确利用益生素和化学益生素，预防肠道疾病的发生和保障仔猪健康。

本文的目的就是描述保育期间伴随开食和断奶仔猪肠道微生物区系的组成和功能及其演替规律，利用体内和体外试验评估大豆素（daidzein）对仔猪肠道微生物区系的可能益生作用，利用实时定量 PCR 技术等基于 16Sr RNA 基因的分子生物学技术研究益生菌株 *Lactobacillus sobrius* S1 对仔猪肠道微生物区系结构和功能的可能有益作用。

全文由逐渐推进的研究性六个章节构成。第二章，综述了大豆异黄酮在动物体内的微生物代谢过程，及其在中国动物生产和肠道微生物区系等方面的作用。第三章，利用 PCR/DGGE 技术对一窝新生腹泻仔猪粪便微生物区系的组成和演替进行了跟踪，以研究开食和断奶对仔猪粪便微生物区系结构及其演替的影响。第四章，应用乳杆菌特异性引物和 PCR/DGGE 技术研究了开食和断奶对仔猪肠道乳杆菌群结构和演替的影响。第五章，体外发酵技术结合乳杆菌特异性引物和 PCR/DGGE 技术研究了大豆素（daidzein）对肠道乳杆菌群结构和数量的影响。第六章，PCR/DGGE 技术研究仔猪口服大豆素（daidzein）后，肠道微生物区系组成的变化。第七章，利用实时定量 PCR 等基于 16S rRNA 基因的分子生物学技术研究了分离自仔猪肠道的益生菌株 *Lactobacillus sobrius* S1 在仔猪口服试验中，对仔猪后段肠道微生物区系组成的影响。

结果显示：1)早期开食有助于稳定仔猪断奶时的微生物区系。 2)伴随着保育期仔猪食物结构的改变仔猪肠道乳杆菌群的结构逐渐演替。开食有助于稳定断奶仔猪乳杆菌群的结构。 *L. reuteri* 和 *L. amylovorus / L. sobrius* 可能是仔猪肠道中永久存在的乳杆菌，而 *L. delbruckii, L. acidophilus* 和 *L. crispatus* 则可能是仔猪肠道中短期存在的乳杆菌。3)体内体外试验均证明大豆素（daidzein）是动物饲料的一种可能化学益生素来源。4) 益生菌株 *Lactobacillus sobrius* S1 能够促进有益菌、抑制病原菌的生长。

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